Molecular analysis of *GJB2* (connexin 26) and *GJB6* (connexin 30) gene mutations in non-syndromic hereditary deafness in South Africa

By

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

ABSTRACT

The most common inherited sensory disorder that affects 1 in 1 000 children is severe hearing loss. In developed countries, about a third of cases have a genetic origin, 80% of which are autosomal recessive forms (DFNB). Before 1993 few genes causing hearing loss had been identified, but since then a large number of genes related to this problem have been identified. Studies indicate that the DFNB1 locus, located at position 13q11-12, contributes to 20% of all childhood deafness and may have a carrier rate as high as 2.8%. There are two genes linked to DFNB1, *GJB2* and *GJB6*, which are the major genetic cause of non-syndromic autosomal recessive deafness. *GJB2* and *GJB6* encode the connexin proteins connexin 26 and 30 (Cx26 and Cx30), respectively.

The specific aim of this study was to determine the role of *GJB2* and *GJB6* in deafness within the South African population, since there are no published results involving South African patients with non-syndromic autosomal recessive deafness. This study therefore involved the identification of mutations within the coding region of the *GJB2* and *GJB6* genes in the South African population and the determination of their specific allele frequencies. Another aim of this study was to analyse the effectiveness of three single-strand conformation polymorphic (SSCP) gel electrophoresis systems in the detection of *GJB2* mutations, for use in a standardised diagnostic program.

A total of 44 families were recruited and divided into either the familial or sporadic study group, which consisted of 16 and 28 families, respectively. Control samples were also screened from 50 Caucasians and 50 Mixed Ancestry individuals collected from the general population. To achieve the aims of this study, polymerase chain reaction (PCR) amplification followed by automated DNA sequencing of the coding regions of *GJB2* and *GJB6* was performed. The three SSCP systems that were tested for their effectiveness in detecting mutations within the coding region of *GJB2* included mini polyacrylamide, SSCP-urea and two buffer gel electrophoresis systems.

In total, six previously reported mutations (35delG, 312del14, W24X, M34T, V37I and W44X), a novel mutation (N62I), and four benign polymorphisms (V27I, A40A, R127H and V153I) were detected in *GJB2*. In the *GJB6* gene only the S199T polymorphism was observed. It was determined that the most common mutations found within the Caucasian and Mixed Ancestry populations of South Africa were 35delG and 312del14 of *GJB2*. An overall detection rate of 35.227% was achieved in non-syndromic autosomal recessive deafness amongst this patient cohort. It was also observed that none of the SSCP gel electrophoresis systems were effective at detecting

all of the *GJB2* mutations. This could change if the systems were specifically optimised for the common mutations that were identified.

This study therefore, provides information that can be used in the formulation of a screening program for non-syndromic autosomal recessive deafness specific to the South African population. Further research should be conducted involving other genes, in addition other population groups of South Africa to provide a more comprehensive genetic diagnostic and counselling tool.

OPSOMMING

Die mees algemene oorerflike sensoriese steuring wat 1 in 1 000 kinders affekteer is ernstige gehoorverlies. In ontwikkelde lande het omtrent een-derde van die gevalle 'n genetiese oorsprong, waarvan 80% outosomaal resessiewe vorms is (DFNB). Tot en met 1993 is min gene wat gehoorverlies veroorsaak geïdentifiseer, maar sedertdien is 'n groot aantal gene gelokaliseer en verskeie is ook al gekloneer. Studies toon dat die DFNB1 loci, wat in posisie 13q11-12 gevind word, 20% van doofheid in kinders veroorsaak, en dit het 'n draer frekwensie van so hoog as 2.8%. Twee gene wat koppeling met DFNB1 toon, *GJB2* en *GJB6*, is die vernaamste genetiese oorsaak van nie-sindromise autosomaal resessiewe doofheid. *GJB2* en *GJB6* koder vir die connexin proteïne 26 en 30 (Cx26 en Cx30), onderskeidelik.

Die spesifieke doel van hierdie studie is om die rol van *GJB2* en *GJB6* in doofheid binne die Suid-Afrikaanse populasie te bepaal, aangesien daar tans nog geen gepubliseerde resultate omtrent Suid-Afrikaanse pasiënte met nie-sindromiese outosomaal resessiewe doofheid is nie. Hierdie studie handel dus oor die identifikasie van mutasies wat binne die koderende areas van die *GJB2* en *GJB6* gene voorkom in die Suid-Afrikaanse populasie, asook oor die bepaling van hulle spesifieke alleel frekwensies. Verder het hierdie studie ten doel om die effektiwiteit van drie enkel-string konformasie polimorfisme (SSCP) gel-elektroforese metodes in die opsporing van *GJB2* mutasies te analiseer met die oog op toekomstige gebruik in 'n gestandardiseerde diagnostiese program.

Altesaam 44 families is ingesamel en gekategoriseer in familiële of sporadiese studie-groepe met 16 en 28 families onderskeidelik. Kontrole monsters van 50 Kaukasiese en 50 Gemengde Herkoms individule uit die algemene populasie is ook getoets. Om die doeleindes van die studie te bereik is PKR amplifikasie en outomatiese DNS volgordebepaling van die koderende area van *GJB2* en *GJB6* gedoen. Die drie SSCP sisteme wat getoets is vir hulle effektiwiteit in die identifisering van mutasies in die koderende area van *GJB2* sluit in mini poli-akrielamied, urea en twee-buffer gel elektroforese sisteme.

In totaal is ses gerapporteerde mutasies (35delG, 312del14, W24X, M34T, V37I en W44X), 'n nuwe mutasie (N62I), en vier onskadelike polimorfismes (V27I, A40A, R127H en V153I) opgespoor in *GJB2*, maar in *GJB6* is net die S199T polimorfisme waargeneem. Uit die resultate kon afgelei word dat 35delG en 312del14 van *GJB2* die mees algemene mutasies binne die Kaukasiese en Gemengde Herkoms bevolkings van Suid Afrika is. Die total ontdekking standaard van 35.227%

vir nie-sindromise autosomaal resessiewe doofheid tussen herdie patient kohort was bereik. Verder is waargeneem dat geen van die SSCP gel elektroforese metodes effektief was om al die mutasies van *GJB2* op te spoor nie. Die situasie kan egter verander as die sisteme spesifiek geoptimiseer word vir die algemene mutasies wat gevind is.

Hierdie studie verskaf dus inligting wat gebruik kan word in die verskaffing van 'n diagnostise program vir nie-sindromise outosomaal resessiewe doofheid wat spesifiek is vir die Suid-Afrikaanse populasie. Verdere navorsing wat ander gene en ander populasie groepe van Suid-Afrika insluit, behoort egter uitgevoer te word om uiteindelik 'n meer uitgebreide genetiese diagnostiese en raadgewing diens daar te stel.

CONTENTS

LIST OF ABBREVIATIONS

Abbreviations are listed in alphabetical order.

α	Alpha
A	Adenine (in DNA sequence)
A	Alanine (Ala)
ABEP	Auditory brainstem evoked potential
AFLP	Amplified fragment length polymorphism
APS	Ammonium persulphate solution: $(NH_4)S_2O_8$
ARMS	Amplification-resistant mutation screening
ASO	Allele specific oligonucleotide
AT-PCR	Arbitrarily primed polymerase chain reaction
β	Beta
BCAR3	Breast cancer anti-estrogen resistance 3 gene
BOR	Branchio-oto-renal syndrome
BSA	Bovine serum albumin
bp	Base pair
c	Centi: 10^{-2}
C	Cytosine (in DNA sequence)
C	Cysteine (Cys)
°C	Degrees centigrade
Ca	Calcium
CEN	Centromere
CDHS	Craniofacial dysmorphism
CFLP	Cleavage fragment length polymorphism
$C_{14}H_{10}BrO_5S$	Bromophenol blue (3',3",5',5"-tetrabromophenolsulfonephthalein)
$C_{25}H_{27}N_2O_6S_2Na$	Xylene cyanol FF
CH_3NO	Formamide (carbamaldehyde)
CH_4N_2O	Urea
C_3H_5NO	Acrylamide
CH_2O_2	Formic acid (hydrogen carboxylic acid)
$C_3H_8O_3$	Glycerol (1,2,3-propanetiol)
$C_7H_{10}O_2N_2$	Bisacrylamide (N,N'-methylene-bis-acrylamide)

CL	Cytoplasmic loop
cM	Centimorgan
cm	Centimetre
CMT	Charcot-Marie-Tooth
CT	C-terminus
Cx26	Connexin 26 protein
Cx30	Connexin 30 protein
Cx31	Connexin 31 protein
Cx30.3	Connexin 30.3 protein
Cx32	Connexin 32 protein
Cx43	Connexin 43 protein
Cx46	Connexin 46 protein
Cx50	Connexin 50 protein
D	Aspartate (Asp)
dB	Decibels
ddH_2O	Double distilled water
ddNTP	2',3'-dideoxynucleotide
Del (or del)	Deletion
DFN	X-linked deafness loci
DFNA	Autosomal dominant deafness loci
DFNB	Autosomal recessive deafness loci
DFNM	Modifying deafness loci
DGI1	Dentinogenesis imperfecta 1
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphate
DPOAEs	Distortion product oto-acoustic emissions
DSCA	Double-stand conformation analysis
dsDNA	Double-stand DNA
DT-PCR	Direct termination polymerase chain reaction
E	Glutamate (Glu)
E1	Extracellular loop 1
E2	Extracellular loop 2
EDTA	Ethylenedediamine tetraacetic acid: $C_{10}H_{16}N_2O_8$
EKV	Erythrokeratodermia variabilis

EMD	Enzymatic mutation detection
ER	Endoplasmic reticulum
EtBr	Ethidium bromide (2,7-diamino-10-ethyl-9phenyl-phenanthridinium bromide): $C_{21}H_{20}BrN_3$
EtOH	Ethanol: CH_3CH_2OH
F	Phenylalanine (Phe)
FM	frequency modulation
FRET	Fluorescence resonance energy transfer
γ	Gamma
g	Gram
G	Guanine (in DNA sequence)
G	Glycine (Gly)
x g	Gravitational acceleration
gDNA	Genomic DNA
GJA1	Gap junction alpha-1 (connexin 43) gene
GJA2	Gap junction alpha-2 (connexin 38) gene
GJA3	Gap junction alpha-3 (connexin 46) gene
GJB1	Gap junction beta-1 (connexin 32) gene
GJB2	Gap junction beta-2 (connexin 26) gene
GJB3	Gap junction beta-3 (connexin 31) gene
GJB4	Gap junction beta-4 (connexin 30.3) gene
GJB6	Gap junction beta-6 (connexin 30) gene
GJIC	Gap junctional intercellular communication
H	Histidine (His)
HA	Heteroduplex analysis
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HED	Hidrotic ectodermal dysplasia (Clouston syndrome)
H_2O	Water
Hz	Hertz
I	Isoleucine (Ile)
IHCs	Inner hair cells
JLNS1	Jarvell and Lange-Nielsen syndrome 1
JLNS2	Jarvell and Lange-Nielsen syndrome 2
K	Lysine (Lys)

K	Potassium
k	Kilo: 10^3
kb	Kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
$KHCO_3$	Potassium hydrogen carbonate
KH_2PO_4	Dihydrogen orthophosphate
λ	Lambda
L	Leucine (Leu)
LINE	Long interspersed nucleotide element
μ	Micro: 10^{-6}
μl	Microlitre
μM	Micromolar
M	Methionine (Met)
M	Molar: moles per litre
M1	Transmembrane domain 1
M2	Transmembrane domain 2
M3	Transmembrane domain 3
M4	Transmembrane domain 4
m	Milli: 10^{-3}
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like-Episodes
MERRF	Myoclonic epilepsy and ragged-red fiber disease
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mm	Millimetre
mM	Millimolar
MnlI	Restriction enzyme with recognition sequence: 5'…CCTC$(N)_7$↓…3', source: an *E. coli* strain containing the cloned *MnlI* gene from *Moraxella nonliquefaciens*
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
n	Nano: 10^{-9}

N	Asparagine (Asn)
NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen orthophosphate
NCBI	National Center for Biotechnology Information, USA
ND	Norrie disease
ng	Nanogram
NH_4Cl	Ammonium chloride
nm	Nanometre
NSHL	Non-syndromic hearing loss
NT	N-terminus
OHCs	Outer hair cells
OI	Osteogenesis imperfecta
OMIM	Online mendelian inheritance in man
%	percentage
p	Pico: 10^{-12}
p	Short arm of a chromosome
P	Proline (Pro)
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline solution
PBT	Piebald trait
PCR	Polymerase chain reaction
PDS	Pendred syndrome
pH	Indicates acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
pmol	Pico mole
q	Long arm of a chromosome
Q	Glutamine (Gln)
R	Arginine (Arg)
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
S	Serine (Ser)
SDS	Sodium dodecyl sulphate: $C_{12}H_{25}NaSO_4$
SHL	Syndromic hearing loss

SINE	Short interspersed nucleotide element
SNHL	Non-syndromic hearing loss
SSCP	Single-strand conformation polymorphism
ssDNA	Single-stand DNA
STL1	Stickler syndrome 1
STL2	Stickler syndrome 2
STL3	Stickler syndrome 3
T	Thymine (in DNA sequence)
T	Threonine (Thr)
Taq polymerase	Deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7, from *Thermus aquaticus* BM, recombinant (*E.coli*)
TBE	Tris borate-EDTA buffer
TEL	Telomere
TEMED	N,N,N,N'-tetramethylenediamine: $C_6H_{16}N_2$
TGN	Trans-golgi network
T_m	Melting temperature
Tris	Tris®: tris(hydroxymethyl)-amino-methane: 2-amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
Tris-HCl	2-amino-2-2(hydroxymethyl)-1,3-propanediol hydrochloride: $C_4H_{11}NO_3.H_2O$
U	Enzyme activity unit
USA	United States of America
USH1B	Usher syndrome type 1B
USH1C	Usher syndrome type 1C
USH1D	Usher syndrome type 1D
USH1F	Usher syndrome type 1F
USH2A	Usher syndrome type 2A
USH3	Usher syndrome type 3
UTR	Untranslated region
UV	Ultraviolet
V	Valine (Val)
V	Volts
VS	Vohwinkel's syndrome
v/v	Volume per volume
W	Tryptophan (Trp)
WS1	Waardenburg syndrome type 1

WS2	Waardenburg syndrome type 2
WS3	Waardenburg syndrome type 3
WS4	Waardenburg syndrome type 4
w/v	Weight per volume
Y	Tyrosine (Tyr)
X	Stop codon

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CHAPTER 1: INTRODUCTION

Severe hearing loss is the most common inherited sensory disorder that affects approximately 0.1% of children worldwide (Kelley *et al.*, 1998). The majority of cases of hearing impairment in developed countries is thought to be due to genetic factors. Approximately 80% of all congenital deafness cases are the result of autosomal recessive inheritance or sporadic mutational events (Estivill *et al.*, 1998).

Studies have indicated that mutations within the gene, *GJB2*, located at position 13q11-12, are major contributors to prelingual, non-syndromic recessive deafness within Caucasian populations (Antoniadi *et al.*, 2000). This gene encodes the gap-junction protein connexin 26 (Cx26), which is expressed in the inner ear and plays an important role in cell signalling by recycling potassium ions (Kelley *et al.*, 1998). More than 70 variants have been identified within the Cx26 gene but some represent benign polymorphisms (Smith and Van Camp, 1999; Kelley *et al.*, 2000). The most common deletion is 35delG, which accounts for approximately 75-80% of all non-syndromic autosomal recessive cases of deafness involving the *GJB2* gene. Other mutations are found in particular populations, such as the 167delT mutation that is found specifically in the Ashkenazi Jewish population at a rate of 4% (Tekin *et al.*, 2001).

It has been reported that the phenotypes due to *GJB2* mutations are variable and mostly independent of the mutation. A possible explanation for this is that there are either environmental factors or modifying genes that can affect the phenotype of a particular *GJB2* genotype (Rabionet *et al.*, 2000). Another interesting phenomenon that has been noticed is that between 10 and 42% of cases of *GJB2* mutations harbour only one mutant *GJB2* allele and even some familial cases that are linked to the DFNB1 locus have no evidence of any mutations in *GJB2*. In both instances it was therefore considered that another connexin gene positioned closely to *GJB2* might be responsible for the non-syndromic autosomal recessive hearing loss phenotype (Del Castillo *et al.*, 2002). The candidate gene was *GJB6*, which encodes the gap junction protein connexin 30 (Cx30). This gene lies within 35 kb of *GJB2* on chromosome 13q12 and is also expressed in the cochlea together with Cx26. These two connexins share 76% similarity, with the major difference being the carboxy terminus of *GJB6* that is 35 amino acid residues longer than that of *GJB2* (Rabionet *et al.*, 2000).

To date few mutations related to non-syndromic recessive deafness have been reported in *GJB6*. A large variant has been documented in the Askenazi Jewish population. It was identified at the 5' end

of *GJB6* and includes the first exon of the gene (Lerer *et al.*, 2001). A second large deletion was discovered in families from Spain and Cuba. This deletion, known as Δ(*GJB6*-D13S1830), crops *GJB6* and extends distally towards *GJB2*, but does not include this gene. This mutation is the second most common mutation in the Spanish population after the 35delG mutation of *GJB2* (Del Castillo *et al*, 2002). A recent report has indicated that the deletions observed in the two previously mentioned studies are identical and involve the removal of approximately 309 kb of genomic DNA (Del Castillo *et al.*, 2003).

The high frequency of the 35delG mutation makes molecular diagnosis and genetic counselling for recessive and sporadic congenital deafness feasible. It has been estimated that screening specifically for 35delG and 167delT mutations of *GJB2* is expected to diagnose up to 50% of familial and 10 - 40% of sporadic cases in the USA and Europe. The identification of the cause of the deafness soon after birth (before six months) allows families to adjust the educational process of the child to improve communication skills that lead to better academic success in the child's future (Estivill *et al.*, 1998; Tekin *et al.*, 2001). In a recent study of cochlea transplantation, the result suggests that screening for *GJB2* mutations may be used as a useful predictor of post-implantation speech acquisition, thereby providing families with better pre-implantation counselling (Fukushima *et al.*, 2002).

A detailed look at the history of the identification of the genes implicated in hearing impairment and the role Cx26 and Cx30 play as gap junctions within the ear is provided in Chapter 2. In this chapter, the role of genetic counselling for non-syndromic recessive hearing loss is also discussed together with the methods that are used for mutation detection. In Chapter 3 the materials and methods employed in this study are presented. Chapter 4 follows with the reporting of the results as well as a discussion of these results. Finally, chapter 5 provides a conclusion of the study together with recommendations for (i) future research and (ii) future provision of a screening program for non-syndromic autosomal recessive deafness in South Africa.

This is the first study to investigate the entire coding region of *GJB2* and *GJB6* for disease related mutations in South Africans who suffer from autosomal recessive or sporadic deafness. This study was conducted to identify known and novel mutations within the *GJB2* and *GJB6* genes and determine their carrier rates in the South African population. The information generated in this study will be used to improve the genetic counselling offered to families and individuals at risk of developing non-syndromic hereditary deafness as well as those already afflicted by this disability.

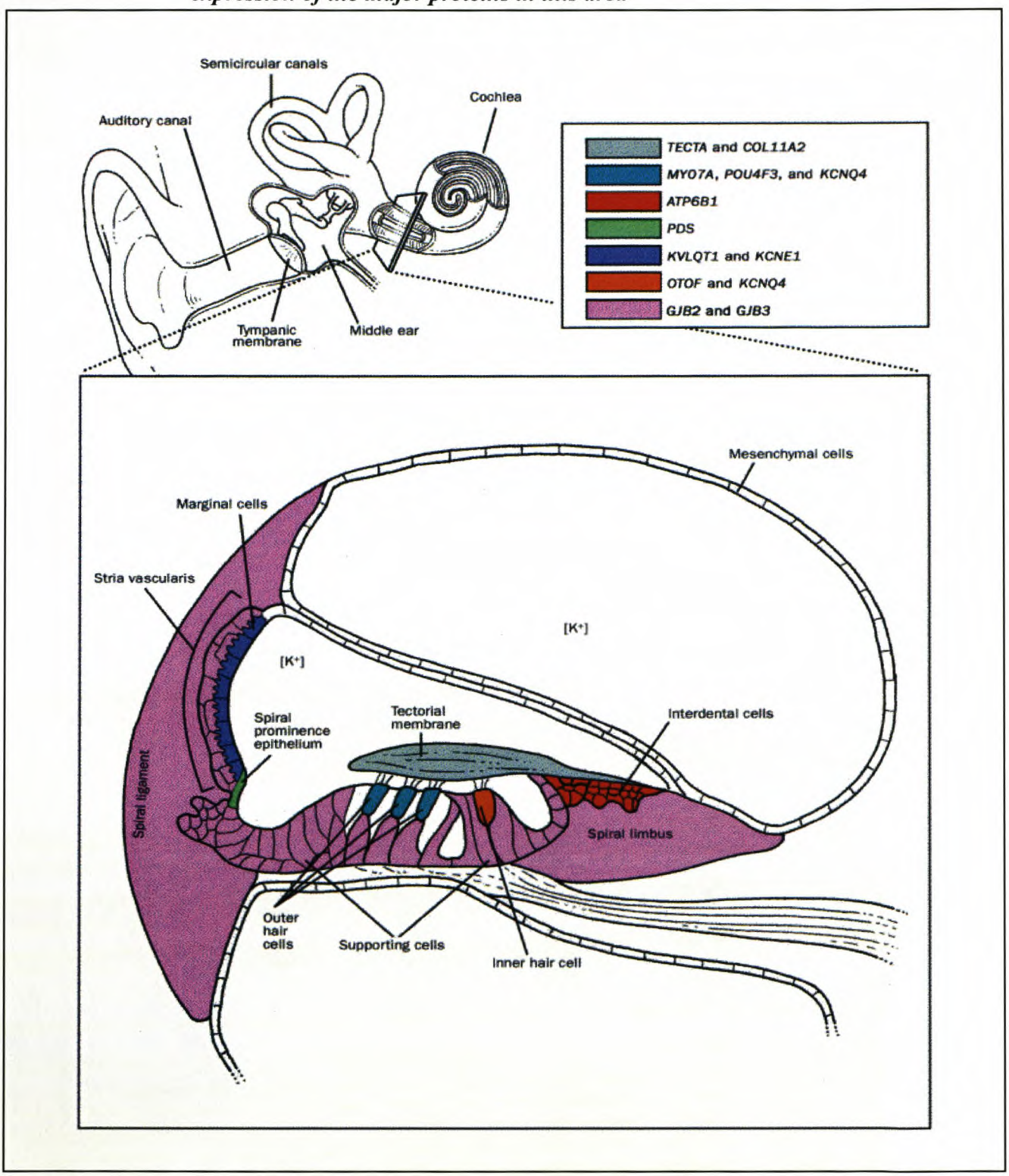
CHAPTER 2: LITERATURE REVIEW

2.1 FUNCTIONING OF THE HUMAN EAR

The ear is divided into three sections, namely outer, middle and inner ear (Figure 2.1). The outer ear is made up of the ear canal and pinna, which is involved in collecting and directing sound. The middle ear includes the eardrum, auditory ossicles and the Eustachian tube, which connects the middle ear to the nasopharynx. The function of the Eustachian tube is to equalize air pressure so that it is the same in the middle ear as it is in the outer ear. If the air pressure is not maintained, the eardrum cannot function properly as it detects small fluctuations in pressure caused by sound waves. Finally, the inner ear comprises the cochlea as well as the mechanisms needed for balance, which includes three connected semicircular canals filled with a fluid known as endolymph (Louw and Edwards, 1995; Mader, 1996).

During the hearing process, sound waves enter the pinna where they are directed down the ear canal, for about 25 mm, until they reach the tympanic membrane, also known as the eardrum. The sound waves are transferred via the tympanic membrane to the inner ear by the auditory ossicles, which consist of three small bones, namely the malleus (hammer), incus (anvil) and stapes (stirrup). This transfer occurs due to the sound waves causing the tympanic membrane to vibrate and these vibrations are detected by the malleus that is attached to the inside of the tympanic membrane. The malleus transfers the vibrations to the incus and finally the stapes, which fits into the vestibule of the inner ear. At the stapes the vibrations are conveyed to the fluid found in the cochlea. Here the vibrations are transformed into nerve impulses, by specialized cells, and are transported along the auditory nerve until they reach the brain (Louw and Edwards, 1995; Mader, 1996).

Figure 2.1: Cross-sectional view of the ear together with the genes responsible for the expression of the major proteins in this area



Adapted from Tekin *et al.*, 2001.

The majority of individuals at some stage will lose some degree of hearing as they age, due to disease, exposure to loud noise or hereditary causes. There are basically three classes of hearing impairment and they are briefly described as follows (Guilford *et al.*, 1994):

- Conductive hearing loss involves a fault in the transferal of sound energy through the outer and middle ear.
- Neurosensory hearing loss involves the incapability of the cochlear to convert the vibrations caused by the sound waves into neural impulses and/or the inability of the auditory nerve to detect or generate these impulses.
- Mixed hearing loss is a combination of both conductive and neurosensory hearing loss.

It is still not clear exactly which areas in the cochlea and the auditory pathway are affected in hearing loss (Engel-Yeger *et al.*, 2002). It is, however, assumed that in the case of deafness due to mutations in connexin proteins, the functioning of gap junctions will be affected. This then leads to an impaired recycling of potassium ions that occurs via the network of gap junctions from the epithelial supporting cells to the cochlear endolymph (Pampanos *et al.*, 2002). This will be discussed in further detail in section 2.4.

2.2 HEARING IMPAIRMENT

2.2.1 CLASSIFICATION OF HEARING LOSS

One infant in 1 000 is affected by hearing impairment and the figure rises to 4% in people under age 45 (Estivill *et al.*, 1998). Hearing loss comprises an entire range of clinical symptoms including congenital versus late onset, conductive versus sensorineural and syndromic versus non-syndromic (Zelante *et al.*, 1997).

The known causes of sensorineural hearing loss can be broadly divided into genetic and environmental causes. The genetic cases can be grouped according to syndromic and non-syndromic or congenital and acquired causes, whereas the environmental cases are caused by viruses, bacteria, trauma, immunology, or a drug-related source as well as other medical conditions (Kenna *et al.*, 2001).

Until recently, genetic forms of deafness were thought to account for only 30% to 45% of cases, with approximately two thirds of these cases being due to isolated, i.e. non-syndromic, genetic forms. The remaining cases were considered to be due to environmental (27% - 35%) or other unidentified causes (Marlin *et al.*, 2001). It has now been recognized that in developed countries, deafness due to an environmental origin has become less frequent due to the improvement in public health conditions. It is now estimated that hearing impairment due to genetic factors may be as high as 59% in some countries (Kemperman *et al.*, 2002; Pampanos *et al.*, 2002).

Hearing impairment that is caused by genetic factors can be divided into syndromic forms, in which the deafness is associated with a number of other abnormalities, and into non-syndromic forms where hearing loss is the only clinical feature (Morell *et al.*, 1998). It has been observed that non-syndromic forms of hearing impairment are usually the more severe as well as being almost exclusively sensorineural in nature, due to cochlear defects. In the case of syndromic forms of deafness, the cause is external and/or middle ear developmental defects that lead to conductive or mixed hearing loss (Pampanos *et al.*, 2002).

Approximately two thirds of inherited hearing loss is non-syndromic, with autosomal recessive inheritance predominating (~80%). The remainder of the cases are characterized by autosomal dominant (~20%), X-linked (~1%) and mitochondrial (<1%) forms (Najmabadi *et al.*, 2002; Pampanos *et al.*, 2002). Due to this high frequency of recessive non-syndromic deafness, it is suggested that a large number of people in the general population are symptom-free carriers of mutations in genes that are involved in hearing (Estivill *et al.*, 1998).

Hearing loss can also be divided according to the age of onset of the deafness. In the case of prelingual hearing impairment it is diagnosed before the age of speech acquisition, therefore before two years of age, whereas postlingual deafness develops at a later stage in life. Generally, it has been observed that recessive deafness tends to be more severe due to the fact that in most cases the hearing loss is profound, prelingual and penetrates fully. In the case of dominant deafness, on the other hand, the hearing loss is often progressive and postlingual, and is frequently diagnosed clinically as unilateral or mild bilateral deafness (Guilford *et al.*, 1994; Denoyelle *et al.*, 1998). This observation may be explained by the fact that in the case of autosomal recessive disorders there is usually a complete

absence of the functional protein. Conversely, in the case of dominant disorders there is usually an initial functioning of the protein followed by a cumulative, degenerative process due to the presence of dominant mutations, which could lead to the subsequent hearing impairment development (Morton, 2002).

2.2.2 GENETIC LOCI INVOLVED IN HEARING IMPAIRMENT

Since 1997 many non-syndromic hereditary forms of deafness have been mapped in the human genome using genetic linkage methodology. The loci are specified as DFNA (autosomal dominant), DFNB (autosomal recessive), DFN (X-linked) or DFNM (modifying) depending on the mode of inheritance of the hearing impairment. The loci are also then numbered consecutively to indicate the order of discovery (Prasad *et al.*, 2000; Kemperman *et al.*, 2002; Morton, 2002).

Amongst the majority of the loci discovered, the underlying disease-causing gene has not been identified (Kemperman *et al.*, 2002). The abundant number of loci involved in non-syndromic deafness attests to the large allelic genetic heterogeneity of this sensory defect (Ben Arab *et al.*, 2000). Most of these genetic types of hearing loss that have been identified are seen quite rarely except for DFNB1 (OMIM: 220290), which has been observed in approximately 40% of children with non-syndromic deafness (Greinwald and Hartnick, 2002; Kemperman *et al.*, 2002).

2.3 DISCOVERY OF GENES IMPLICATED IN HEARING IMPAIRMENT

Even though the mechanism of hearing impairment has been studied for many years it is only recently that the genetic background in the form of the genes and molecular system underlying this disorder has begun to be unearthed (Morton, 2002). Whilst the genetic basis of non-syndromic deafness is still at the first stages of discovery, the identification of genes responsible for syndromic deafness is well developed. This is primarily related to the problems faced in locating genes due to the fact that non-syndromic deafness is an extremely heterogenic disorder (Guilford *et al.*, 1994).

To date approximately 70 loci have been identified. 28 have been linked to recessive, 33 to dominant, three to both dominant and recessive, five to X-linked, and two to mitochondrial hearing impairment

(Kenna *et al.*, 2000; Pampanos *et al.*, 2002). Of the 70 loci, at least 58 genes involved in the hearing process have been distinguished. Of these 58 genes, 34 have been linked to non-syndromic deafness of which, 16 are autosomal dominant, 11 are autosomal recessive, one is X-linked and six are mitochondrial genes (Table 2.1). A further 24 genes have been discovered that are responsible for syndromic deafness (Morton, 2002). It has been suggested from population studies that as many as 100 genes may be involved in non-syndromic deafness (Guilford *et al.*, 1995; Morell *et al.*, 1998; Lalwani 2002 and Shahin *et al.*, 2002).

Table 2.1: Documented genes that play a role in the auditory system

No.	Gene	Protein or RNA	Map Position	Syndromic or non-syndromic hearing loss	Disorders
1	ATP6B1		2cen-q13	SHL	Distal renal tubular acidosis associated with sensorineural deafness
2	BSND	Barttin	1p32.3	SHL	Bartter syndrome
3	CDN23	Cadherin 23	10q21-q22	NSHL + SHL	Usher syndrome type 1D (USH1D); Usher syndrome type 1D (USH1D) + DFNB12
4	CLDN14	Claudin 14	21q22	NSHL	DFNB29
5	COCH	Cochlin	14q12-q13	NSHL	DFNA9
6	COL1A2	Collagen type 1 α2	7q22.1	SHL	Osteogenesis imperfecta
7	COL2A1	Collagen type 2 α1	12q13.1-q13.2	SHL	Stickler syndrome (STL1)
8	COL4A3	Collagen type 4 α3	2q36-q37	SHL	Alport syndrome
9	COL4A4	Collagen type 4 α4	2q36-q37	SHL	Alport syndrome
10	COL4A5	Collagen type 4 α5	Xq22	SHL	Alport syndrome
11	COL11A1	Collagen type 11 α1	1p21	SHL	Stickler syndrome (STL2)
12	COL11A2	Collagen type 11 α2	6p21.3	NSHL + SHL	Stickler syndrome (STL3); DFNA13
13	DDP		Xq22	SHL	Mohr-Tranebjaerg syndrome
14	DFNA5		7p15	NSHL	DFNA5
15	DIAPH1	Diaphanous	5q31	NSHL	DFNA1
16	DSPP	Dentin sialophosphoprotein	4q21.3	SHL	Dentinogenesis imperfecta 1 (DGI1); DFNA39

17	EDN3	Endothelin 3	20q13.2-q13.3	SHL	Waardenburg-Shah syndrome (WS4)
18	EDNRB	Endothelin receptor B	13q22	SHL	Waardenburg-Shah syndrome (WS4)
19	EYA1		8q13.3	SHL	Branchio-oto-renal syndrome
20	EYA4		6q22-q23	NSHL	DFNA10
21	GJA1	Connexin 43	6q21-q23.3	NSHL	Autosomal recessive deafness
22	GJB2	Connexin 26	13q12	NSHL	DFNB1
23	GJB3	Connexin 31	1p34	NSHL	DFNA2
24	GJB6	Connexin 30	13q12	NSHL	DFNA3 and DFNB1
25	KCNE1		21q22.1-q22.2	SHL	Jervell and Lange-Nielsen syndrome (JLNS2)
26	KCNQ4		1p34	NSHL	DFNA2
27	KVLQT1		11p15.5	SHL	Jervell and Lange-Nielsen syndrome (JLNS1)
28	MITF		3p12.3-p14.1	SHL	Waardenburg syndrome type II (WS2); Tietz syndrome
29	MYH9	Myosin heavy chain 9	22q13	NSHL + SHL	May-Hegglin and Fechtner syndromes; DFNA17
30	MYO6	Myosin 6	6q13	NSHL	DFNA22
31	MYO7A	Myosin 7A	11q12.3-q21	NSHL + SHL	Usher syndrome type 1B (USH1B); DFNA11; DFNB2; Atypical Usher syndrome
32	MYO15A	Myosin 15A	17q11.2	NSHL	DFNB3
33	NDP	Norrin	Xp11.3	SHL	Norrie disease
34	OTOF	Otoferlin	2p22-p23	NSHL	DFNB9
35	PAX3		2q35	SHL	Waardenburg syndrome type I (WS1); Waardenburg syndrome types I + III (WS1 + WS3); Craniofacial dysmorphism (CDHS)
36	PCDH15	Protocadherin 15	10q21-q22	SHL	Usher syndrome type 1F (USH1F)
37	POU3F4		Xq21.1	NSHL	DFN3
38	POU4F3		5q31	NSHL	DFNA15
39	PMP22	Peripheral myelin	17p11.2	SHL	Charcot-Marie-Tooth disease
40	12S rRNA	Ribosomal RNA	mitochondrial	NSHL	Sensorineural deafness

41	SLC19A2		1q23.3	SHL	Thiamine-responsive megaloblastic anemia with diabetes mellitus and deafness
42	SLC26A4	Pendrin	7q31; 7q21-q34	NSHL + SHL	Pendred syndrome (PDS); DFNB4
43	SOX9		17q24.3-q25.1	SHL	Campomelic dysplasia
44	SOX10		22q13	SHL	Waardenburg-Shah syndrome (WS4)
45	STRC	Stereocilin	15q21-q22	NSHL	DFNB16
46	TCOF1	Treacle	5q32-q33.1	SHL	Treacher Collins syndrome
47	TECTA	Alpha tectorin	11q22-q24	NSHL	DFNA8, DFNA12, DFNB21
48	TMC1		9q13-q21	NSHL	DFNA36, DFNB7/B11
49	TMPRSS3		21q22.3	NSHL	DFNB8, DFNB10
50	tRNA-glu	Transfer RNA	mitochondrial	SHL	Diabetes and deafness
51	tRNA-leu	Transfer RNA	mitochondrial	SHL	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS); Diabetes mellitis and deafness
52	tRNA-lys	Transfer RNA	mitochondrial	SHL	Myoclonic epilepsy and ragged-red fiber disease (MERRF)
53	tRNA-ser	Transfer RNA	mitochondrial	SHL	Retinitis pigmentosum and progressive sensorineural hearing loss
54	tRNA-ser(UNC)	Transfer RNA	mitochondrial	NSHL + SHL	Sensorineural deafness; Progressive myoclonic epilepsy, ataxia and hearing loss; Palmoplantar keratoderma and deafness
55	USH1C	Harmonin	11p15.1	SHL	Usher syndrome type 1C (USH1C)
56	USH2A	Usherin	1q41	SHL	Usher syndrome type 2A (USH2A)
57	USH3		3q21-q25	SHL	Usher syndrome type 3 (USH3)
58	WFS1	Wolframin	4p16	SHL + NSHL	Wolfram syndrome; DFNA38; DFNA6/A14

Adapted from Morton, 2002.

2.3.1 METHODS USED FOR GENE IDENTIFICATION

Due to hearing impairment being a very heterogeneous disorder it was thought that deafness was a complex problem and therefore not much research was conducted. However, this complication, especially when it comes to counseling and prevention, is no longer such a big problem in view of the following factors (Zelante *et al.*, 1997):

- Non-syndromic recessive deafness accounts for the majority (70 - 75%) of all cases of genetic deafness.
- Up to 80% of all non-syndromic recessive hearing loss is linked to DFNB1.
- A single frameshift mutation, 35delG, found in DFNB1 accounts for most of the hearing loss associated with non-syndromic recessive deafness in the European Caucasian population.

Conventional methods, such as genetic linkage analysis, that were used for locating other disease genes were not successful for the identification of genes related to deafness because of its complexity. For non-syndromic recessive deafness, linkage analysis was hindered due to family size, as it is necessary to use large consanguineous families or populations that have not been affected by immigration. For non-syndromic dominant deafness single, small families are sufficient. Another factor that complicated analysis is the possibility of more than one deafness gene being present in a given family or that the hearing loss was in fact due to a non-genetic cause (Guilford *et al.*, 1994; Brown *et al.*, 1996; Morton 2002).

Other methods for gene discovery had to be developed and it appears that positional cloning in humans has been the most successful to date with a number of new genes relating to non-syndromic recessive deafness being identified (Brown *et al.*, 1996). Another method that was used, was the identification of candidate genes from humans and mice as well as others animals (Morton 2002). This was used in organ and tissue-specific techniques, which involved the construction of cochlear cDNA libraries from humans and mice. These were subsequently used to identify possible genes by conducting mutation screens, as a transcript map of the inner ear is a source of positional candidate genes. Candidate genes were also used in the development of mouse models, as they are the ideal animal models for studying hearing impairment. Mouse models were constructed for specific types of hearing loss so that research

could be done on the developmental time points involved in the disorder by studying the animals at various stages of development from fetus to adult (Table 2.2). Even with all the progress that has occurred in the identification of the genes involved in hearing loss, there are still relatively few mouse models available (Brown *et al.*, 1996; Morton, 2002).

Table 2.2: Mouse models of hearing impairment and their human orthologs

Mouse mutant	Gene	Human disorder(s)
Ames waltzer (av)	Pcdh15	Usher syndrome type 1F (USH1F)
Beethoven (Bth) and deafness (dn)	Tmc1	DFNA36, DFNB7/B11
Chondrodysplasia (cho)	Col 11a1	Stickler syndrome type 2 (STL2)
Col 1a1 transgene disruption	Col 1a1	Osteogenesis imperfecta (OI)
Col 11a2 targeted null	Col 11a2	Stickler syndrome type 3 (STL3), DFNA13
Col 4a3 targeted null	Col 4a3	Alport syndrome
Disproportionate micromelia (Dmm) and mutant transgenes	Col 2a1	Stickler syndrome type 1 (STL1)
Dominant megacolon (Dom)	Sox10	Waardenburg-Shah syndrome (WS4)
Dominant spotting (W)	Kit	Piebald trait (PBT)
Eya1^{bor} and targeted null	Eya1	Branchio-oto-renal syndrome (BOR)
Fgfr3 targeted null	Fgfr3	Craniosynostosis
Gata targeted null	Gata3	Hypoparathyroidism, sensorineural deafness and renal dysplasia syndrome (HDR)
Kcne1 targeted null and Kcne1^{pkr}	Kcne1	Jervell and Lange-Nielsen syndrome (JLNS2)
Kcne1 targeted null	Kcnq1	Jervell and Lange-Nielsen syndrome (JLNS1)
Lethal spotting (ls)	Edn3	Waardenburg-Shah syndrome (WS4)
Microphthalmia (mi)	Mitf	Waardenburg syndrome type 2 (WS2); Tietz syndrome
Ndph targeted null	Ndph	Norrie disease (ND)
Pax2 targeted null	Pax2	Renal-coloboma syndrome
Piebald (s)	Ednrb	Waardenburg-Shah syndrome (WS4)
Pou3f4 targeted null and sex-linked fidget (slf)	Pou3f4	DFN3
Pou4f3 targeted null and dreidel (ddl)	Pou4f3	DFNA15
Quivering (qv)	Spnb4	Charcot-Marie-Tooth disease type 4F (CMT4F)
Shaker-1 (sh1)	Myo7a	Usher syndrome type 1B (USH1B); DFNB2; DFNA11; atypical Usher syndrome
Shaker-2 (sh2)	Myo15a	DFNB3
Slc26a4 targeted null	Slc26a4	Pendred syndrome (PDS); DFNB4
Snell's waltzer (sv)	Myo6	DFNA22

Splotch (Sp)	Pax3	Waardenburg syndromes types 1 and 3 (WS1; WS3); Craniofacial dysmophism, hand abnormalities, profound sensorineural deafness (CDHS)
Tecta targeted null	Tecta	DFNA8/A12; DFNB21
Thrd targeted null	Thrb	Thyroid hormone resistance
Tremble (Tr)	Pmp22	Charcot-Marie-Tooth disease type 1A (CMT1A)
Waltzer (v)	Cdh23	Usher syndrome type 1D (USH1D); DFNB12

Adapted from Morton, 2002.

Finally, a number of other techniques have also been tried, such as autozygosity mapping. The term autozygosity in this context implies homozygosity for markers that are exactly alike by descent, therefore inherited from a recent common ancestor. This method involves looking at consanguineous families, with non-syndromic autosomal recessive deafness, that are probably autozygous for markers that are linked to this disease locus. This approach is a very powerful tool for linkage analysis if families containing a number of affected individuals in two or more sibships that are linked by inbreeding can be found (Strachan and Read, 1999).

2.3.2 THE IDENTIFICATION AND LOCALIZATION OF DFNB1

The most important discovery connected with non-syndromic hearing loss was the identification of the DFNB1 locus by Guilford *et al.* (1994). This was the first locus to be described that was linked to autosomal recessive deafness. Three years later the gene, *GJB2*, linked to the DFNB1 locus was mapped to position 13q12 and it was discovered that it encoded the gap junction β-2 protein, known as connexin 26 (Cx26). To date, more than 60 mutations relating to non-syndromic deafness have been identified within *GJB2* (Connexin Homepage, 2003). However, it is interesting to note that one particular mutation, 35delG, is particularly common, especially amongst Caucasian populations (Jun *et al.*, 2000; Kenna *et al.*, 2001).

DFNB1 was first identified by linkage analysis using two consanguineous families from Tunisia that demonstrated autosomal recessive non-syndromic deafness. This was further confirmed by the observation of other consanguineous families, from different ethnic groups, with recessive non-syndromic deafness. A number of non-consanguineous Caucasian families were also subsequently

linked to several markers from chromosome 13q12-13 (Scott *et al.*, 1998A; Ben Arab *et al.*, 2000; Pampanos *et al.*, 2002).

Subtractive hybridization was used to identify Cx26. This was achieved by the selection for mRNAs that were expressed in normal mammary epithelial cells, but were not present in mammary tumor cell lines (Kelley *et al.*, 2000). Kikuchi *et al.* (1995) conducted immunostaining research on the expression of Cx26 in the rat cochlea. He discovered that staining occurred in non-sensory epithelial cells that included the interdental cells of the spiral limbus, inner sulcus cells, supporting cells of the organ of Corti, outer sulcus cells, and the cells found within the root processes of the spiral ligament. He also found that staining occurred in the connective tissue cells of the cochlea that took into account the mesenchymal cells that line the scala vestibuli, different types of fibrocytes of the spiral limbus and spiral ligament, as well as the basal and intermediate cells of the stria vascularis. This information was used together with data that was gathered from *in vitro* mutagenesis and physiological studies to link Cx26 with the development of hearing impairment.

Shortly after identifying *GJB2* as a possible candidate gene responsible for autosomal non-syndromic recessive deafness, a number of mutations were detected within *GJB2* in three Pakistani families that had been linked to 13q11. This was followed by the detection of mutations in *GJB2* among many other families that originated from Italy, Spain and Israel. Thereby, the *GJB2* gene was confirmed as a major contributor to non-syndromic, autosomal recessive deafness in Caucasian populations (Pampanos *et al.*, 2002).

Since then it has been demonstrated that the DFNB1 locus is also responsible for a second type of non-syndromic deafness, which is a dominant form known as DFNA3 (OMIM: 601544) (Guilford *et al.*, 1995; Denoyelle *et al.*, 1998). It has been determined that *GJB2* (Section 2.5) and *GJB6* (Section 2.6) are both responsible for DFNB1 as well as autosomal dominant non-syndromic hearing loss (DFNA3) even though they appear as two different forms of hearing impairment with totally contrasting characteristics (Table 2.3).

Table 2.3: Mutations in **GJB2** *that result in non-syndromic dominant hearing impairment*

Dominant Mutations			
Mutation name	**Description**	**Effect**	**Protein domain**
delE42	deletion of AGG at 125	deletion of Glutamic acid at 142	E1
W44S	G to C at 131	Tryptophan at 44 into Serine	E1
W44C	G to C at 132	Tryptophan at 44 into Cysteine	E1
R75Q	G to A at 224	Arginine at 75 into Glutamine	E1
R184Q	G to A at 551	Arginine at 184 into Glutamine	E1
C202F	G to T at 605	Cysteine at 202 into Phenylalanine	E1

Adapted from the Connexin Homepage, 2003.

2.4 GAP JUNCTIONS AND CONNEXINS

2.4.1 GAP JUNCTIONS AND THEIR ROLE IN HEARING

In animal tissue, when cells are adjoining each other, they are often connected by gap junction structures that produce a widespread intercellular signaling mechanism, known as gap junctional intercellular communication (GJIC). Therefore, GJIC allows for the passive flow of small molecules between adjacent cells thereby connecting cells metabolically (Garrett and Grisham, 1999; Ahmad and Evans, 2002). Gap junctions promote the fast exchange of intercellular ions and other molecules, which results in almost immediate action potentials being produced. Due to this property, gap junctions are found in neurons and other excitable cells, such as epithelial cells as well as smooth and cardiac muscle cells (Zelante *et al.*, 1997; Prasad *et al.*, 2000). Gap junctions are also found in cells that are not directly connected with the circulatory system, such as the lens cells of the eye, where GJIC provides a means of nutrient transportation (Garrett and Grisham, 1999).

Gap junctions are formed from hexameric arrangements of specific proteins, known as connexins. Each sub-unit of the arrangement, known as a connexon or hemichannel, is made up of six connexins that form a cylinder, with an average length of 7.5 nm and diameter of 2.5 nm. In this conformation the connexons are usually tilted, with regards to the sixfold axis running down the center of the hexamer, and bridge the extracellular space between the two neighboring plasma membranes. This configuration produces a central pore with an average diameter of approximately 1.8 to 2.0 nm, which allows small

molecules of up to 1.2 kDa to pass through uninhibited, but prevents proteins, nucleic acids and other large structures from doing so (Garrett and Grisham, 1999; Müller *et al.*, 2002). This central pore is an aqueous channel that joins the cytoplasm of the adjacent cells and forms the primary structural unit of the gap junctions that are found as closely packed groups (Krutovskikh and Yamasaki, 2000; Ahmad and Evans, 2002).

A number of coordinated events have to take place for GJIC to assemble, whereby it is assumed that different parts of the connexin molecule are implicated in regulating the various steps of the procedure. Firstly, the newly formed connexin protein must be inserted into the membrane of the endoplasmic reticulum (ER) accurately, where it is then assembled into a connexon. Secondly, the connexon has to be correctly separated from the Golgi apparatus, which is then followed by its transportation into the lateral plasma membrane of the cell. Finally, it has to find and dock with a correlating connexon in an adjacent cell thereby producing an intracellular channel (Krutovskikh and Yamasaki, 2000).

It is important to be able to regulate metabolic intercellular communication, hence the gap junctions have the ability to close under abnormal conditions thereby protecting neighbouring cells. This closure of the central pore occurs by a twisting, sliding motion of the connexons produced by a localized conformation change at the cytoplasmic ends of the connexins. Gap junctions appear to be sensitive to membrane potentials, hormonal signals, pH changes and intracellular calcium (Ca) ion levels. However, it has been suggested that Ca ions play an important role in the gating of the gap junction. Under normal conditions the intracellular levels of Ca ions is less than 10^{-7} M, thereby signaling the opening of gap junctions so that intracellular communication can take place. On the other hand, the gap junctions are quickly closed if the Ca ion level reaches 10^{-5} M or higher (Garret and Grisham, 1999; Müller *et al.*, 2002).

One of the most important functions of gap junctions is the regulation of the fluid and ion balance within the cochlea, which is a unique system that allows for the separation of ions as well as their transfer and transport during the process of hearing. This communication, which occurs via gap junctions between bordering basal cells, sustains the endolymphatic potential by retaining the high levels of potassium (K). This is achieved by removing K ions, which were released from the cochlear hair cells into the extracellular space within the organ of Corti, by way of the gap junction network of the epithelial cells. The K ions are then discharged in an area where the extracellular K concentration is

lower. An example of a location with a lower extracellular K concentration would be the root cell process. A brief summary of the process would be as follows: a mechanical vibration of the cochlear leads to K ions entering the hair cell where it is then released basolaterally via the epithelial gap junctions back to the stria vascularis (Engel-Yeger *et al.*, 2002).

A number of research results have supported the vital role that gap junctions play in the auditory process. Firstly, it has been shown that there are gap junctions present between the outer hair cells (OHCs) and the supporting cells, which include melanocytes, within the ear. Secondly, the endothelium of the scala media of the cochlea, which is separated from the endolymphatic space by tight junctions in the marginal cell layer, has also been implicated in the manufacturing of a receptor response to sound. Finally, gap junctions have been identified in both epithelial and connective tissue cells, which are involved in the recycling of endolymphatic K ions, in the rat cochlea. This provides a morphological basis for GJIC taking place in response to an auditory stimulus as well as for the electric coupling of receptor cells (Zelante *et al.*, 1997).

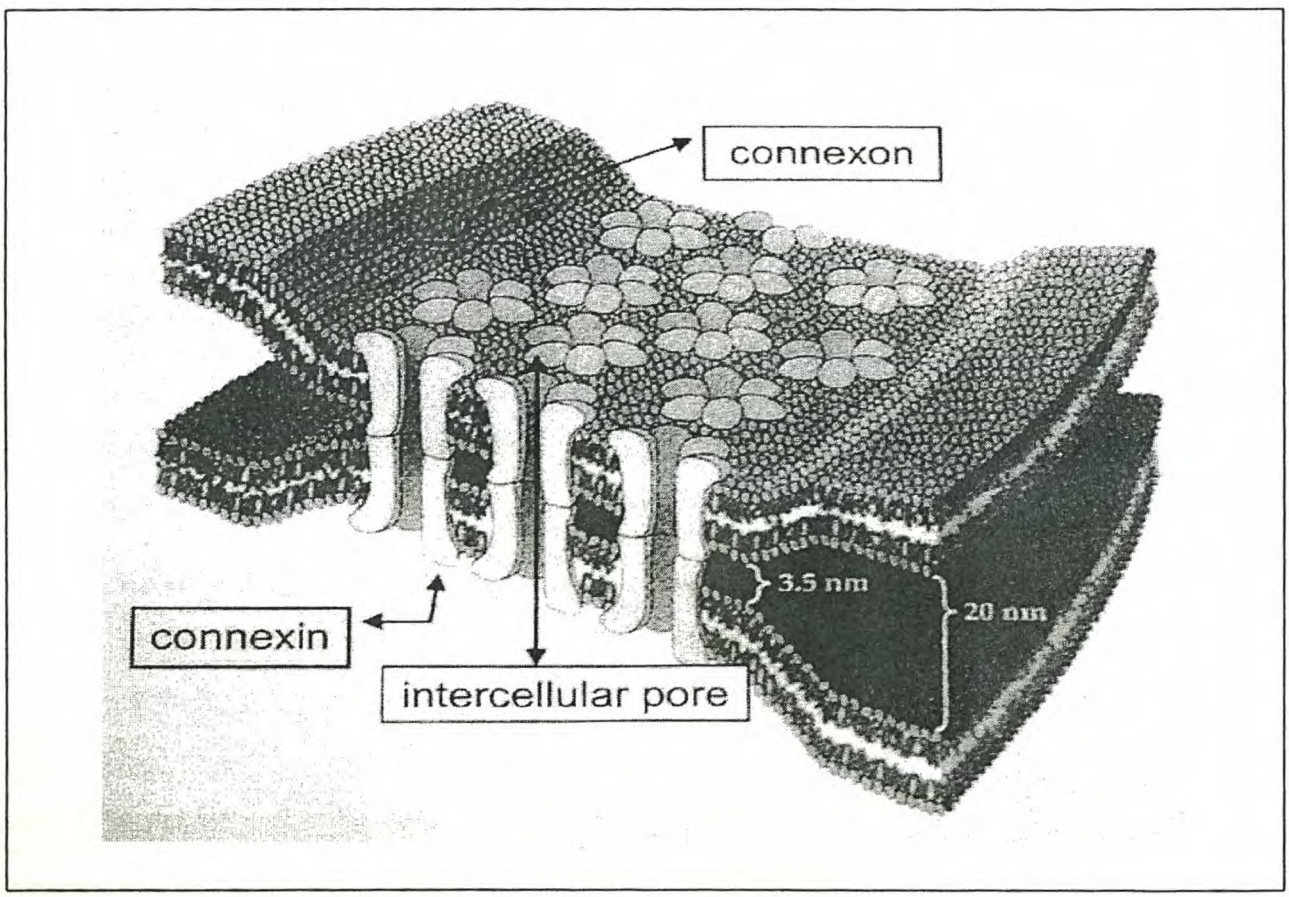
There is some debate as to how a mutation in the connexin that forms the gap junctions would affect the auditory process. According to Engel-Yeger *et al.* (2002) the disrupted recycling of K ions would deprive the stria vascularis of K ions because they would not have been released into the scala media where it would act as the dominant cation involved in carrying the hair cell receptor currents. Therefore, the impaired recycling of K ions may hamper the establishment of the receptor potential that is found in both the OHCs, which are sensitive to the displacement of the basilar membrane, and the inner hair cells (IHCs), which are sensitive to the basilar membrane velocity. On the other hand, Lefebvre and Van De Water (2000) maintain that the disturbed recycling of K ions would lead to the local intoxication of the organ of Corti. This would occur due to the K ions not being removed from the synapses at the base of hair cells and being transported via the supporting cells and fibroblasts to the endolymph in the cochlear duct.

2.4.2 THE CONNEXIN PROTEIN FAMILY

Connexin proteins are involved in the formation of gap junctions that are found in glial, epithelial, smooth and cardiac muscle cells (Estivill *et al.*, 1998). Six connexin protein chains form a hexamer, known as a connexon, which then links with another connexon in a neighboring cell to form a gap

junction (Figure 2.2). A connexon can be made up of a number of different types of connexins, and a variety of connexons can link to form various types of gap junctions. Therefore, linking of the same connexons produces homotypic channels whereas when different connexons bond they result in heterotypic channels. This ability allows gap junctions to have different distinct physiological properties depending on the tissue they are present within, thereby determining which molecules or ions can pass through (Jun *et al.*, 2000; Kemperman *et al.*, 2002).

Figure 2.2: Schematic representation of a gap junction



Adapted from Kemperman *et al.*, 2002.

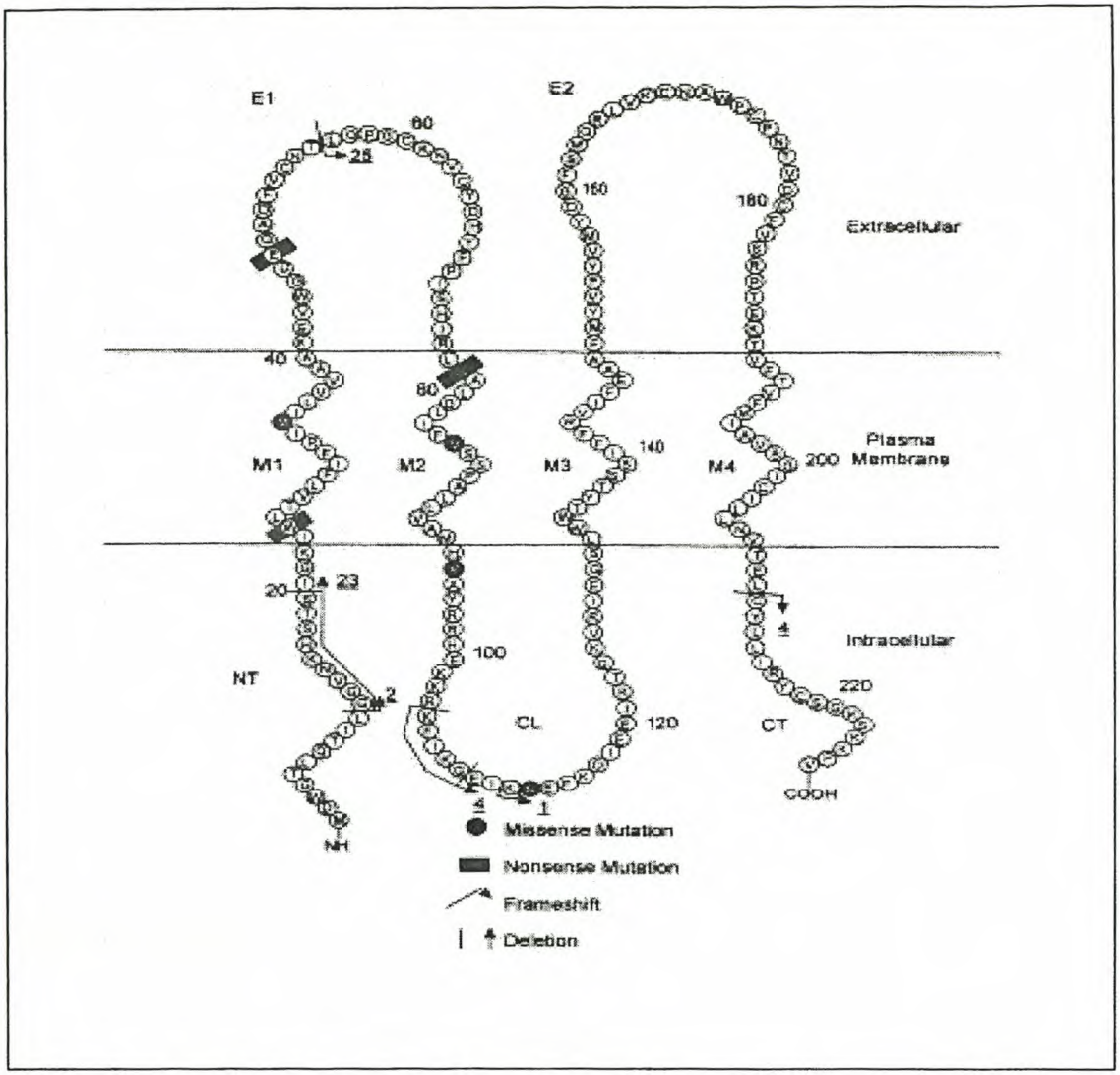
To date, 21 members of the connexin family have been discovered in mammals as well as mammalian isoforms in non-mammalian vertebrates (Müller *et al.*, 2002). The family can be divided into at least three subfamilies, α, β, and γ, according to evolutionary properties. However, it is more common to name the different connexin proteins according to their molecular weights that vary from 25 – 62 kDa (Krutovskikh and Yamasaki, 2000; Ahmad and Evans, 2002). Often the connexin genes are found as

clusters on a chromosome. For example, genes for connexins 26, 30 as well as 46 are found on chromosome 13q12 and it is thought that a fourth possible connexin gene may also be reside in the same area.

Gap junctions are found as thousands of linked connexon pairs that are arranged into junctional plaques that are planar, double-layered structures with clear borders. Connexins are membrane proteins that display a conserved core that contains the intracellular N-terminus, four transmembrane domains and two disulphide-linked extracellular loops. They also contain a variable section that is made up of the cytoplasmic loop and intracellular C-terminus (Maestrini *et al.*, 1999; Müller *et al.*, 2002). Each area of the connexin protein has a specific role to play in its correct functioning (Figure 2.3), which is briefly summarized below (Kelley *et al.*, 1998; Krutovskikh and Yamasaki, 2000):

- N-terminus (NT): is relatively small, approximately 20 residues. It plays a role, together with M1, in voltage gating of the central pore of gap junctions.
- Transmembrane domains (M1 – M4): are thought to play a role in the formation of the central pore and therefore they are important in the selective permeability of the gap junction. It is also thought that these areas are crucial for the correct transportation of the connexin into the plasma membrane.
- Extracellular loops (E1 and E2): are crucial for the correct linking of neighbouring connexons. There are three cystines present, which are conserved among all the connexin family, that are important for intramolecular stabilization. These areas are also involved in the selective compatibility between different connexin types when forming heterotypic channels.
- Cytoplasmic loop (CL): is not highly conserved as it has a variable size and amino acid composition amongst the connexin family. Together with the C-terminus it is implicated in pH gating of the gap junctional channel.
- C-terminus (CT): is the most variable area amongst the connexin family and it has been suggested that it is not important for normal functioning.

Figure 2.3: Schematic representation of Cx26 and some of its mutations



Adapted from Kelley *et al.*, 1998.

The structure and function of connexins have been studied in great detail at the biochemical and physiological levels by using mutant forms. It appears that connexins may be unique amongst other integral multimeric membrane proteins, as it seems that they do not oligomerize within the ER but in the trans-Golgi network (TGN) instead, where an unknown TGN chaperone may be involved. This is a novel mechanism that is similar to that which is observed in the production of channels by a number of bacterial toxin proteins, therefore it is implied that this is an evolutionary conserved membrane-integration mechanism that has been retained in mammals. It has been suggested that the levels of most of the different connexin types are linked to transcription or mRNA turnover as it seems to be rapid (Kelley *et al.*, 1998; Ahmad and Evans, 2002).

The mutation studies of connexin proteins have also highlighted a number of conserved residues, which indicate that these residues are very important for the correct functioning of the connexin no matter what tissue-specific role it is in. There appears to be some evidence that connexin proteins also play a role in other functions besides GJIC, such as the regulation of the expression of growth control genes, cell differentiation, as well as the suppression of tumors (Krutovskikh and Yamasaki, 2000). This was shown by Kartz (1995) who proposed that connexin expression might play a role in the production of complex functional architecture during the development of the brain by preparing the basis of cortical circuits that will mature by synapse formation. It was also shown that *GJB2* is considered a class II tumor-suppressor gene because it is down regulated in tumor tissue, whereas it is up regulated during the S and G2 phases of the cell cycle (Kelley *et al.*, 1998).

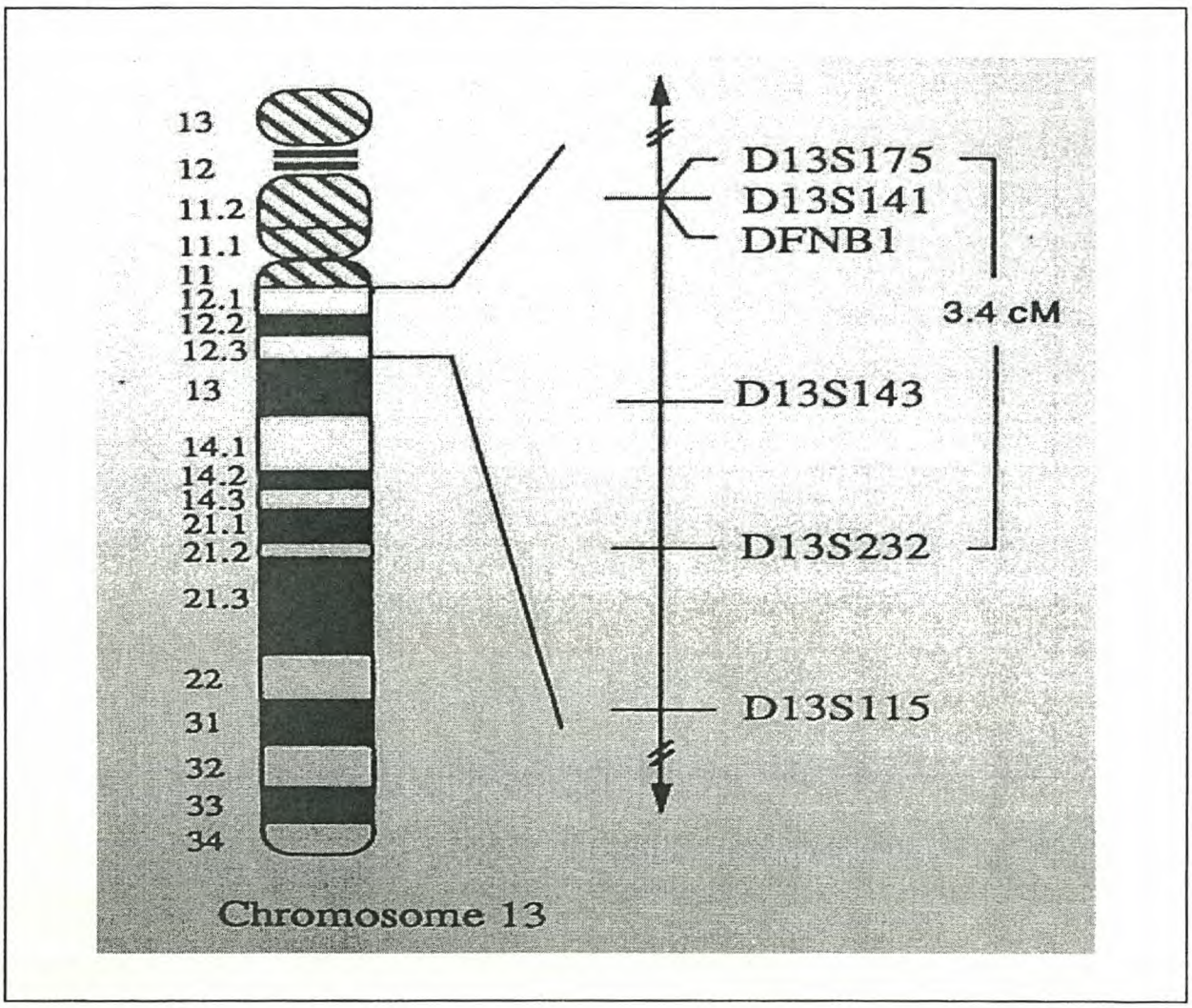
It was also discovered during mutation analysis studies that several mutations that had been detected in different connexin genes, and were involved in different diseases, were in fact topologically similar. In addition, all of the mutations were either in the transmembrane domains (mostly in M2) or situated close to them. It is interesting to note that even though the expression of a mutated connexin occurs in many tissue types, because all connexin mutations that lead to human diseases are caused by germ-cell mutations, only certain functions of organs or tissue properties are affected. For example, the majority of the mutations found in *GJB2* lead to the complete absence of Cx26. However, the only clinical symptom that develops is deafness, even though Cx26 is expressed in a number of different types of tissue. It is suggested that this is possible because other connexins can act as a substitute for Cx26 in the other tissues but not in the cochlea (Estivill *et al.*, 1998; Krutovskikh and Yamasaki, 2000). It appears that the connexin protein family plays an integral part in the process of normal hearing as it has been discovered that a number of mutations found in different connexins have been associated with hearing loss (Lin *et al.*, 2001).

2.5 THE CONNEXIN 26 PROTEIN

In 1994 the first autosomal recessive non-syndromic deafness locus, DFNB1, on chromosome 13q11 was identified by Guilford *et al.* This was followed in 1997 by the discovery of the gene, *GJB2* (OMIM: 121011), that is linked to this locus (Figure 2.4). *GJB2* belongs to a highly conserved family

of genes that encode the polypeptides that make up gap junctions. More specifically *GJB2* encodes Cx26, which is a β-2 gap junction protein that is expressed in distinct areas of the cochlea (Griffith *et al.*, 2000; Jun *et al.*, 2000; Houseman *et al.*, 2001).

Figure 2.4: A schematic representation of chromosome 13 indicating the position of the DFNB1 locus together with polymorphic markers

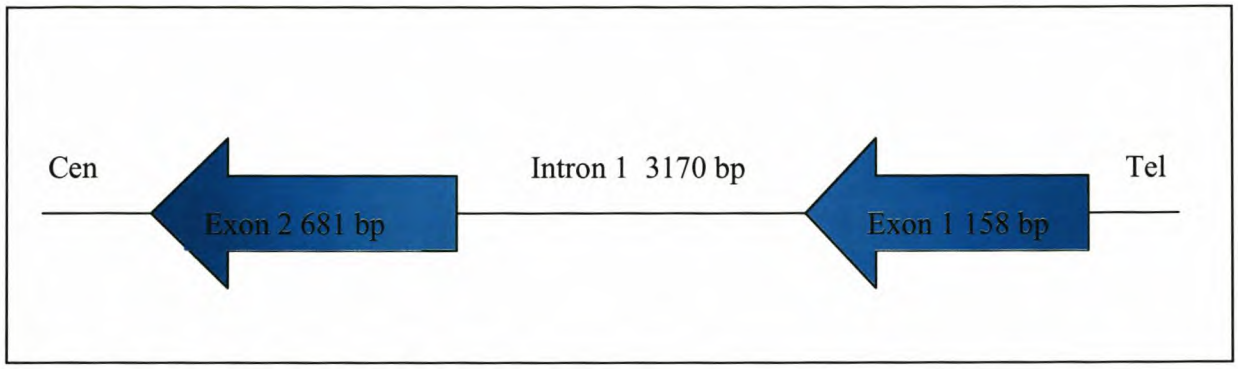


Adapted from Scott *et al.*, 1995.

The *GJB2* gene is small with a length of approximately 5.5 kb of which only 2.4 kb is mRNA that translates into a protein consisting of 226 amino acids. The gene consists of two exons separated by a single intron. The first exon is non-coding whereas the second exon codes for the Cx26 protein (Figure 2.5). The size of the non-coding exon 1 is 158 bp and its 3' end is situated 3170 bp upstream from the

single intron. The first exon is non-coding whereas the second exon codes for the Cx26 protein (Figure 2.5). The size of the non-coding exon 1 is 158 bp and its 3' end is situated 3170 bp upstream from the 5' end of the coding exon 2 whose size is approximately 681 bp (Kiang *et al.*, 1997; Denoyelle *et al.*, 1999; Kemperman *et al.*, 2002; Pampanos *et al.*, 2002).

Figure 2.5: Diagram of* GJB2 *showing the single coding exon (Exon 2) and the non-coding exon (Exon 1)

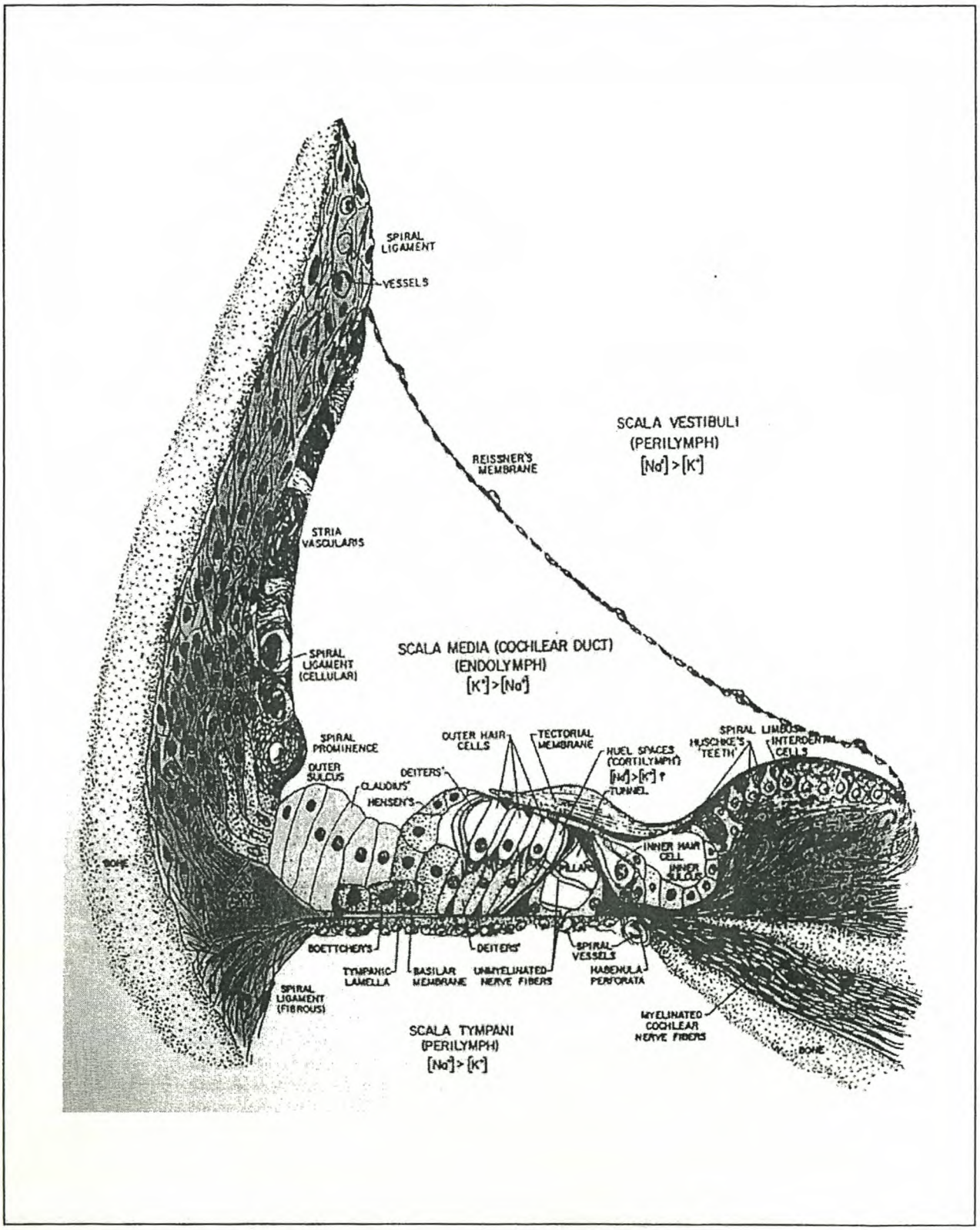


Adapted from Ferraris *et al.*, 2002.

2.5.1 FUNCTIONS OF CONNEXIN 26

Cx26 gap junctions are found together with other connexins in numerous types of cells and tissues in humans including alveolar cells, ependyma hepatocytes, intestine, keratinocytes, leptomeninges, myometrium, pancreatic acinar cells and pinaelocytes (Scott *et al.*, 1998A; Denoyelle *et al.*, 1999). The main area where Cx26 is expressed, as gap junctions, is in the inner ear within the cochlea non-sensory epithelial and the connective tissue cells (Engel-Yeger *et al.*, 2002.). The non-sensory epithelial cells include the inner sulcus cells, interdental cells of the spiral limbus, supporting cells of the Organ of Corti, outer sulcus cells, and the cells found within the root process of the spiral limbus (Figure 2.6). On the other hand, the connective tissue cells include the fibrocytes of the spiral limbus and spiral ligament, basal and intermediate cells of the stria vascularis as well as the mesenchymal cells found lining the scala vestibuli (Kammen-Jolly *et al.*, 2001; Kenna *et al.*, 2001).

Figure 2.6: Structure of the cochlea indicating the various non-sensory epithelial cells and connective tissue cells



Adapted from Jun *et al.*, 2000.

While studying the immunoreactivity traits of Cx26 within rats the distribution of Cx26 was discovered. The first indication of Cx26 activity is at the prenatal age of 11 weeks where it is then maintained at a high level for 31 weeks of gestation. By fetal week 20 the distribution of Cx26 activity appears similar to that of adults. It was found that the emergence of the Cx26 activity followed in parallel with the start of development and histological maturation. It was also established that Cx26 activity plays a role in functional maintenance (Kammen-Jolly *et al.*, 2001).

Due to the presence of Cx26 within the different types of cells found in the inner ear, it was believed that it is involved in the recycling of K ions during the process of hearing. This was assumed due to the fact that gap junctions are necessary for maintaining the high K concentration found in the endolymph of the inner ear (Kenna *et al.*, 2001; Kemperman *et al.*, 2002). It is understood that if there is a loss in functioning of Cx26 molecules it will affect the proper working of gap junctions. This leads to a disturbance in the flow of K ions through the network of gap junctions being produced, which will lead to hearing loss occurring (Van Laer *et al.*, 2001).

2.5.2 THE FREQUENCY OF DFNB1 LINKED DEAFNESS

It is estimated that one in 3750 children in the general population is affected by autosomal recessive non-syndromic deafness due to mutations occurring in the *GJB2* gene. This figure was determined by taking the accepted figures of one in 1000 children suffering from congenital hearing loss, together with the two thirds of these that are due to a genetic cause. Also taken into account was the 80% that have an autosomal recessive pattern of inheritance and the approximate half that are thought to be due to mutations in *GJB2*. From this it could also be determined that the carrier frequency for *GJB2* mutations is approximately 3.3% or one in 30 (Wilcox *et al.*, 1999). However, the actual contribution to non-syndromic hearing impairment by mutations found in the *GJB2* gene varies from 0 – 40 % between populations thereby demonstrating genetic heterogeneity (Pampanos *et al.*, 2002).

High carrier rates of specific mutations, such as seen with those found in *GJB2*, are normally ascribed to a founder effect that took place sometime in the past within the given population. To prove this hypothesis one can study the conservation of haplotypes with the use of closely linked markers. In the case of recessive genes, the high carrier rates are usually caused by bottlenecks taking place within a

population, which causes the size of the population to reduce greatly. This leads to the conservation of the haplotype around the mutation due to there being very few mutant chromosomes present in the population when the bottleneck occurs (Morell *et al.*, 1998).

The high carrier rate can also be caused by endogamy, which is the bias of individuals to marry within their community. For example, if all deafness was due to recessive genes at a single locus and all those that were deaf were to marry each other, it would lead to the frequency of hearing loss to double within the first generation. At first it was assumed that this phenomenon would not play such a large role due to hearing impairment being heterogenic, but due to the discovery that hearing loss due to *GJB2* mutations is more common than previously thought, it had to be reconsidered. This is due to the fact that in a population where random mating occurs the expected frequency of homozygotes would be the square of the gene frequency for a specific type of recessive hearing loss. However, in the case of marriages between two homozygotes with the same deafness, the frequency would equal to the fourth power of the gene frequency (Nance *et al.*, 2000).

2.5.3 THE CHARACTERISTICS OF AUTOSOMAL RECESSIVE NON-SYNDROMIC HEARING LOSS INVOLVING MUTATIONS IN *GJB2*

The phenotypic features that have been linked to autosomal recessive non-syndromic hearing loss due to mutations in *GJB2* include that the deafness is prelingual in onset, and has no associated syndromic conditions or radiological abnormalities of the inner ear. There should also be no vestibular irregularities, which would include walking taking place after 18 months, episodes of vertigo and abnormal vestibular caloric test result (Jun *et al.*, 2000; Marlin *et al.*, 2001).

The degree of hearing impairment that is observed can be classified as mild, moderate, severe and profound. However, it was noted that there was a characteristic loss amongst the high frequencies (4 000 – 10 000 Hz). Generally, the hearing loss was not progressive and it was associated with sloping or flat audiometric curves, which were equal in both ears (Denoyelle *et al.*, 1999; Wilcox *et al.*, 2000).

Specifically, for 35delG homozygotes it has been observed that there is no distortion product oto-acoustic emissions (DPOAEs) except sporadically at 1 000, 8 000 and 10 000 Hz. Whereas, those individuals with normal hearing have larger DPOAE responses at all frequencies than those individuals

who are carriers of the 35delG mutation. With auditory brainstem evoked potential (ABEP) tests it is noticed that homozygotes have variable results but the majority show partial or absent ABEPs. This therefore indicates that the function of the outer hair cells (OHCs) is affected by the 35delG mutation (Engel-Yeger *et al.*, 2002).

Another characteristic of autosomal recessive non-syndromic deafness is interfamilial as well as intrafamilial variation in the severity of the hearing impairment. This means that there is a difference in the degree of hearing loss amongst families, generations as well as siblings. A possible reason for this is that other factors affect the expression of the mutated *GJB2* gene, for example, a second gene that shares partial functioning together with *GJB2* (Engel-Yeger *et al.*, 2002). More specifically this variation in the level of deafness could be caused by the variable expression of this second gene or its polymorphic variants. Alternatively, the variation could be explained by the timing or the level of expression of Cx26 or a second gene (Kelley *et al.*, 1999).

Clinical evaluation at a microscopic level of temporal bones of those suffering from non-syndromic autosomal recessive deafness revealed that no neural degeneration is present and that there is a healthy collection of spiral ganglion cells. However, there is an almost complete deterioration of hair cells in the organ of Corti, agenesis of the stria vascularis, as well as a severed and rolled-up tectorial membrane. The condition of the tectorial membrane is possibly due to the near total lack of hair cells in this area of the inner ear. Due to the neurons in the spiral ganglion still being preserved it is possible to conduct successful cochlear implants for the rehabilitation of deaf patients with DFNB1 linked hearing impairment (Jun *et al.*, 2000).

2.5.4 MUTATIONS OF *GJB2* THAT ARE INVOLVED IN DFNB1 HEARING IMPAIRMENT

Even though up to a 100 different genes have been identified that play a role in hearing impairment it is now accepted that mutations in *GJB2* account for up to 40% of all genetic cases. (Nance *et al.*, 2000) Altogether more than 70 mutations of *GJB2* have been linked to deafness (Table 2.4), most of which are found in the coding region of *GJB2* encoded by a single exon of 681 bp (Simsek *et al.*, 2001; Shahin *et al.*, 2002). It is assumed that those mutations that lead to missense, deletion or frameshift changes are likely to be inactivating null mutations, which would lead to the formation of proteins that are either non-membrane anchored or are missing their cytoplasmic tails (Carrasquillo *et al.*, 1997). It

has been estimated that the carrier frequency of any mutation found in the *GJB2* gene is most likely between 1:20 and 1:25 (Estivill *et al.*, 1998).

***Table 2.4: Sequence variations reported in* GJB2**

Autosomal recessive mutations that cause non-syndromic recessive deafness			
Mutation name	**Description**	**Effect**	**Protein domain**
IVS1+1 G to A	G to A at –3172	Splice site	None
-3170G to A	G to A at –3170	Splice site	None
M1V	A to G at 1	No protein production	NT
T8M	C to T at 23	Threonine into Methionine	NT
31del14	deletion of 14 nucleotides at 31	Frameshift	NT
31del38	deletion of 38 nucleotides at 31	Frameshift	NT
G12V	G to T at 35	Glycine at 12 into Valine	NT
35delG	deletion of G at 30-35	Frameshift	NT
35insG	insertion of G at 30-35	Frameshift	NT
K15T	A to C at 44	Lysine at 15 into Threonine	NT
51del12insA	deletion of 12 nucleotides and insertion of A at 51	Frameshift	NT
S19T	G to C at 56	Serine at 19 into Threonine	NT
I20T	T to C at 59	Isoleucine at 20 into Threonine	NT
W24X	G to A at 71	Tryptophan at 24 into a stop codon	M1
V27I+E114G	G to A at 79 + A to G at 341	Valine at 27 into Isoleucine and Glutamic acid at 114 into Glycine	M1 + CL
R32C	C to T at 94	Arginine at 32 into Cysteine	M1
R32H	G to A at 95	Arginine at 32 into Histidine	M1
M34T	T to C at 101	Methionine at 34 into Threonine	M1
V37I	G to A at 109	Valine at 37 into Isoleucine	M1
A40E	C to A at 119	Alanine at 40 into Glutamic acid	E1
W44X	G to A at 132	Tryptophan at 44 into a stop codon	E1
G45E	G to A at 134	Glycine at 45 into Glutamic acid	E1
E47X	G to T at 139	Glutamic acid at 47 into a stop codon	E1
E47K	G to A at 139	Glutamic acid at 47 into Lysine	E1
167delT	deletion of T at 167	Frameshift	E1
Q57X	C to T at 169	Glutamine at 57 into a stop codon	E1
176-191del16	deletion of 16 nucleotides at 176	Frameshift	E1
Y65X	C to G at 195	Tyrosine at 65 into a stop codon	E1
W77R	T to C at 229	Tryptophan at 77 into Arginine	M2

W77X	G to A at 231	Tryptophan at 77 into a stop codon	M2
235delC	deletion of C at 233-235	Frameshift	M2
L79P	T to C at 236	Leucine at 79 into Proline	M2
Q80P	A to C at 239	Glutamine at 80 into Proline	M2
I82M	C to G at 246	Isoleucine at 82 into Methionine	M2
V84L	G to C at 250	Valine at 84 into Leucine	M2
S85P	T to C at 253	Serine at 85 into Proline	M2
L90P	T to C at 269	Leucine at 90 into Proline	M2
269insT	insertion of T at 269	Frameshift	M2
V95M	G to A at 283	Valine at 95 into Methionine	CL
Y97X	not described	Tyrosine at 97 into a stop codon	CL
290-291insA	insertion of A at 290	Frameshift	CL
H100Y	C to T at 298	Histidine at 100 into Tyrosine	CL
299-300delAT	deletion of AT at 299-300	Frameshift	CL
302del3	deletion of AGA at 302	Deletion of Lysine at 102	CL
E101G	A to G at 302	Glutamic acid at 101 into Glycine	CL
310del14	deletion of 14 nucleotides at 310	Frameshift	CL
312del14	deletion of 14 nucleotides at 312	Frameshift	CL
314del14	deletion of 14 nucleotides at 314	Frameshift	CL
333-334delAA	deletion of AA at 333-335	Frameshift	CL
S113R	T to G at 339	Serine at 113 into Arginine	CL
delE120	deletion of GAG at 360	deletion of Glutamic acid at 119-120	CL
K122I	A to T at 365	Lysine at 122 into Isoleucine	CL
Q124X	C to T at 370	Glutamine at 124 into a stop codon	CL
Y136X	C to A at 408	Tyrosine at 136 into a stop codon	CL
S139N	G to A at 416	Serine at 139 into Glutamine	CL
R143W	C to T at 427	Arginine at 143 into Tryptophan	M3
E147K	G to A at 439	Glutamic acid at 147 into Lysine	M3
469delG	deletion of G at 469	Frameshift	E2
486insT	insertion of T at 486	Frameshift	E2
R165W	C to T at 493	Arginine at 165 into Tryptophan	E2
504insAAGG	insertion of AAGG at 504	Frameshift	E2
509del14	deletion of 14 nucleotides at 509	Frameshift	E2
509insA	insertion of A at 509	Frameshift	E2
515del17	deletion of 17 nucleotides at 515	Frameshift	E2
W172X	G to A at 516	Tryptophan at 172 into a stop codon	E2
P175T	C to T at 523	Proline at 175 into Threonine	E2
V178A	T to C at 533	Valine at 178 into Alanine	E2

R184W	C to G at 550	Arginine at 184 into Tryptophan	E2
R184P	G to C at 551	Arginine at 184 into Proline	E2
572delT	deletion of T at 572	Frameshift	E2
S199F	C to T at 596	Serine at 199 into Phenylalanine	M4
I203K	TC to AA at 608	Isoleucine at 203 into Lysine	M4
N206S	A to G at 617	Glutamine at 206 into Serine	CT
631delGT	deletion of GT at 631-632	Frameshift	CT
L214P	T to C at 641	Leucine at 214 into Proline	CT
645-648delTAGA	deletion of TAGA at 645	Frameshift	CT

Mutations with unknown modes of inheritance

Mutation name	Description	Effect	Protein domain
IVS1-12C>T	C to T at -12 from exon 2	Unknown	None
V84A	T to C at 251	Valine at 84 into Alanine	M2
T123N	C to A at 368	Threonine at 123 into Asparagine	CL
E129K	G to A at 385	Glutamic acid at 129 into Lysine	CL
R143Q	G to A at 428	Arginine at 143 into Glutamine	M3
Y155X	T to A at 465	Threonine at 155 into a stop codon	M3
M163V	G to A at 487	Methionine at 163 into Valine	E2
A171T	G to A at 511	Arginine at 171 into Threonine	E2
A197S	G to T at 589	Alanine at 197 into Serine	M4

Mutations of unknown effect (polymorphisms)

Polymorphism	Description	Frequency	Protein domain
Exon1-493del10	del of 10 nt at 493 5' of exon 1	not determined	5' UTR
-3558T to C	T to C at -3558	not determined	5' UTR
C/T at -15	C to T at -15	not determined	5' UTR
V27I	G to A at 79	not determined	M1
I30I	T to A or C at 90	not determined	M1
A40A	A to C at 120	not described	E1
S72C	C to G at 215	not determined	E1
F83L	C to G at 249	3/200 control chromosomes	M2
L89L	C to A at 267	not determined	M2
E114G	A to G at 341	not determined	CL
T123A	A to G at 367	not determined	CL
R127H	G to A at 380	not described	CL
I128I	C to T at 384	1%	CL
V153I	G to A at 457	4/367 controls (1%)	M3
G160S	G to A at 468	2/200 control chromosomes	E2
C169Y	G to A at 506	not determined	E2

V182V	G to ? at 546	not determined	E2
V190V	C to T at 570	not described	E2
I203T	T to C at 608	not determined	M4
C/T at 682	C or T at 682	not determined	3' UTR
C/T at 765	C or T at 765	not determined	3' UTR

Adapted from the connexin homepage, 2003.

It has been reported in some studies that not all individuals suffering from hearing loss carry both copies of the *GJB2* mutation that are necessary for them to exhibit recessive deafness (Marlin *et al.*, 2001; Lalwani, 2002). There are a number of possible explanations for this phenomenon. Firstly, there could be variants in the non-coding region that could influence the gene expression of *GJB2*. This however, is unlikely because further research in this area has not identified any such mutations. Secondly, the single *GJB2* mutation may only be present by chance and not be related at all to hearing loss, however this explanation seems unlikely. Finally, the most likely explanation is that another gene could be affecting the capability of *GJB2* from functioning normally (Wilcox *et al.*, 2000).

Interestingly, the *GJB2* gene has also been implicated in disorders besides autosomal recessive non-syndromic deafness: six mutations have been linked to DFNA3 and three have been linked to syndromic disorders. These disorders include an autosomal dominant skin disease known as Clouston hidrotic ectodermal dysplasia (HED, OMIM: 129500) and Vohwinkel's syndrome (VS, OMIM: 124500), which is characterized by mutilating keratoderma and hearing loss (Kelsell *et al.*, 1997; Maestrini *et al.*, 1999; Kenna *et al.*, 2001). This phenomenon of more than one disorder being linked to the same region of a chromosome is possibly due to either the existence of two closely linked genes, or a single mutation within a single gene presenting different symptoms depending on the genetic background or other environmental factors. The most likely explanation is that different mutations within the same gene produce different symptoms depending where they lie within the protein itself, thereby, having an effect on the protein's structure or function (Scott *et al.*, 1995).

It has been reported that different mutations of *GJB2* play an important role in different populations, for example, 35delG in Caucasians, 167delT in Ashkenazi Jews, 235delC in Asians and R143W in Africans (Pampanos *et al.*, 2002). These four specific mutations will be discussed in further detail in the following sections.

2.5.4.1 THE 35delG MUTATION

The *GJB2* mutation, 35delG, is formed due to a deletion of one guanine (G) from a set of six that are located from nucleotide position 30 to 35. This leads to a frameshift mutation resulting in a premature stop codon at nucleotide 38 (Pampanos *et al.*, 2002). The Cx26 protein that is formed is significantly shorter than in the wild type protein, and therefore it is unlikely to be inserted into the plasma membrane.

This mutation is the most common mutation causing non-syndromic autosomal recessive deafness in Caucasian populations. The frequency of 35delG varies from greatly between ethnic groups such as 0.0 in Japan and 0.313 in Lebanon. The carrier frequency within the Mediterranean is estimated to be as high as 1:31, however, it is lower or non-existent in other ethnic groups (Table 2.5). It can be deduced from both the carrier frequency of *GJB2* mutations as well as the prevalence of other mutations besides 35delG in non-Caucasian populations that different variations will characterize those which are common to these ethnic groups (Rickard *et al.*, 2001).

Table 2.5: Carrier frequency of the 35delG mutation within several populations

Population	Carrier frequency
Northern and central Europe	
Norway	0.005
Denmark	0.021
Estonia	0.044
United Kingdom	0.119
Germany	0.050
Belgium	0.012
Holland	0.022
France (Brittany)	0.010
France	0.103
Czech Republic	0.021
Slovenia	0.005
Austria	0.101
Bulgaria	0.006

Southern Europe	
Portugal	0.022
Spain	0.023
Italy	0.122
Italy (Sardinia)	0.034
Malta	0.028
Greece	0.121
Turkey	0.116
North America	
White Americans	0.019
African Americans	0.0
Asian Americans (Indian, Japanese, Koreans)	0.0
Ashkenazi Jewish Americans	0.007
Other populations	
Egypt	0.0
Arabs	0.017
Lebanon	0.313
Israel	0.147
Korea	0.005
Japan	0.0
Australia	0.041
Oman	0.0

Adapted from Van Laer *et al.*, 2001; Kemperman *et al.*, 2002; Pampanos *et al.*, 2002.

There is a debate as to whether the high frequency of the 35delG mutation is due to a founder effect or the presence of a mutational hotspot. There appears to be an equal amount of evidence supporting both possibilities. A fluctuation in carrier frequencies for the 35delG mutation between different ethnic groups together with the presence of other common mutations in these populations support the hypothesis that the high frequency is due to a founder effect (Pampanos *et al.*, 2002). If the theory that the high frequency is due to a hypermutable region were correct, it would be expected that all ethnic groups would have had roughly the same carrier frequency (Abe *et al.*, 2000). Furthermore, haplotype analysis has revealed the sharing of a very small chromosomal interval between patients that are homozygous for the 35delG mutation, thereby indicating that the common ancestor occurred approximately 500 generations or roughly 10 000 years ago (Shahin *et al.*, 2002). Conversely, the information supporting the hypothesis that the high frequency is due to a mutational hotspot includes that this nucleotide sequence has structural similarities to other known hypermutable regions within the

genome. Further evidence is provided by the deletion of any of the six Gs resulting in the same genotype (Kelley *et al.*, 2000; Tekin *et al.*, 2001).

If it is assumed that the high frequency of 35delG is due to a founder effect it is then possible to explain the spread of this mutation across Europe, the Mediterranean, the United States as well as other countries due to the age of this variation. It was hypothesized that the mutation probably originated somewhere in the Middle East from where it radiated throughout Europe along the two Neolithic population movement lines, the first of which reached Italy and Spain by following the coast of the Mediterranean Sea, whereas the second reached Northern Europe by following the Danube and Rhine valleys. From Europe the mutation would have spread to the United States and other countries by means of colonization and emigration (Van Laer *et al.*, 2001).

2.5.4.2 THE 167delT MUTATION

Mutation 167delT in *GJB2* is caused by a deletion of a Thymine (T) at nucleotide 167, which leads to a frameshift mutation to occur at amino acid 56, followed by a premature termination after a further 25 codons (Sobe *et al.*, 1999). The Cx26 peptide that is formed is largely truncated and therefore it is not expected to assemble correctly or show any functional activity as a gap-junction protein (Griffith *et al.*, 2000). From analyzing the haplotypes that flank the 167delT mutation it was inferred that it had originated from a single founder as there was a conservation of this region amongst patients (Morell *et al.*, 1998; Tekin *et al.*, 2001).

The 167delT mutation is the second most common mutation worldwide, but it is confined to the Ashkenazi Jewish population at a carrier frequency of approximately 4% (Tekin *et al.*, 2001). From this high carrier frequency it has been roughly estimated that 1 in 1765 Asheknazi Jews are affected by autosomal recessive non-syndromic deafness (Morell *et al.*, 1998).

2.5.4.3 THE 235delC MUTATION

The range of mutations in *GJB2* found within the Asian population is considerably different from those that are found in other ethnic groups with an European background. For example, the 35delG mutation of *GJB2* is not observed in the Japanese population, however, the 235delC mutation is seen in the

Japanese but not the Caucasian population (Abe *et al.*, 2000, Kudo *et al.*, 2000). The 235delC mutation is caused by a deletion of a Cytosine (C) at nucleotide position 235, which leads to a frameshift that is followed by a premature stop codon.

The 235delC mutation is the most common variant in Asia with a frequency amongst those suffering from autosomal recessive non-syndromic deafness ranging from 1% in Japan to 5% in Korea (Park *et al.*, 2000; Houseman *et al.*, 2001). These high frequencies together with the fact that they are limited to a specific ethnic background suggests that the 235delC mutation is the result of a founder effect, similar to the 167delT mutation which occurs in the Ashkenazi Jewish population (Abe *et al.*, 2000).

2.5.4.4 THE R143W MUTATION

In Ghana the R143W mutation in *GJB2* was identified at a frequency of 90%, making it the most common *GJB2* mutation linked to autosomal recessive non-syndromic hearing loss in that population (Hamelmann *et al.*, 2001). This high frequency is most likely due to a founder effect, because haplotype analysis has revealed that the region around the mutation has been conserved and the mutation probably occurred 60 generations ago (Brobby *et al.*, 1998).

The R143W mutation is caused by the substitution of a T by a C base at nucleotide position 427, which leads to the exchange of amino acids from a Tryptophan to an Arginine at codon 143. This mutation therefore falls in the M3 domain of the Cx26 protein, which would influence the selective permeability of the central pore (Brobby *et al.*, 1998). Interestingly, when comparing the pattern of Ghananian *GJB2* mutations to other populations, a number of differences are noted (Hamelmann *et al.*, 2002):

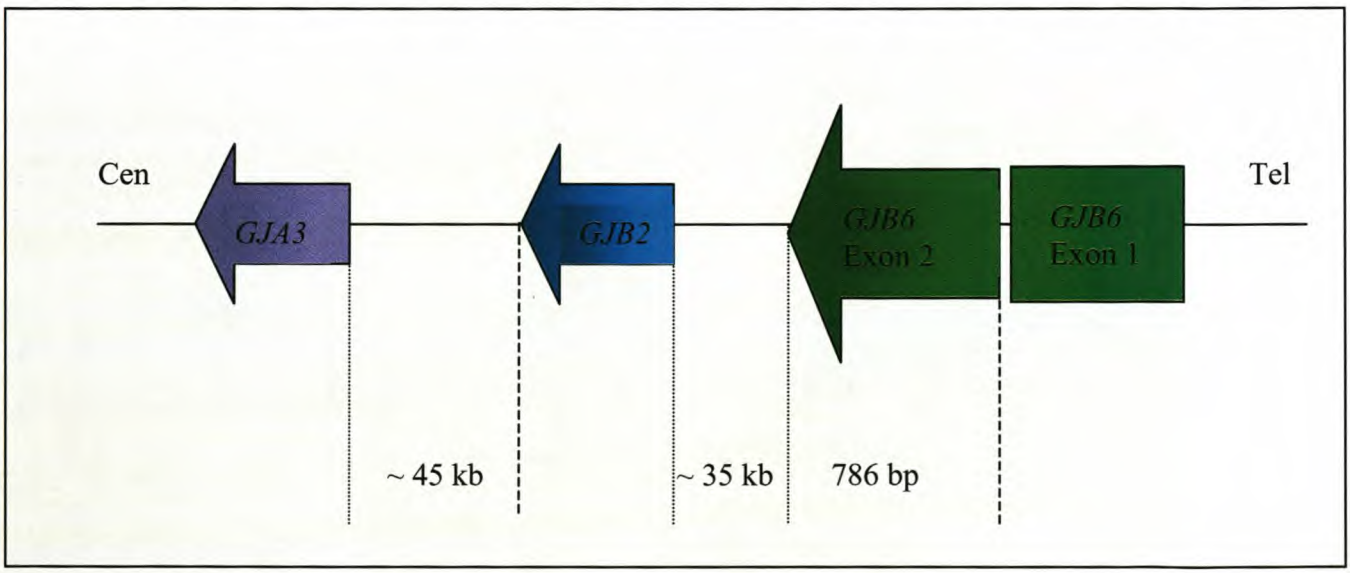
- Compared with European countries where mutations in *GJB2* cause 30-50% of all deafness in childhood, in Ghana this is only true for 16%. The reason for this could be due to other causes having a greater influence, such as meningitis that has a higher incidence rate in Africa.
- Nonsense and frameshift mutations found within *GJB2* predominate around the world but they are very rare in the Ghanaian population.
- Mutations tend to cluster in the C-terminal half of the Cx26 protein within the Ghanaian population whereas in other populations the majority of mutations are found in the N-terminal half.

2.6 THE CONNEXIN 30 PROTEIN

While screening families with autosomal recessive non-syndromic deafness it was noticed that there were some cases where the DFNB1-linked family members did not exhibit the expected *GJB2* mutations. This lead to the suggestion that another gene in the same region could be responsible for these cases as only about 75% of the predicted mutations were detected (Grifa *et al.*, 1999; Kelley *et al.*, 1999). The candidate gene that was suspected of also playing a role in autosomal recessive non-syndromic hearing loss was connexin 30 (Cx30) as Lauterman *et al.* (1998) had discovered the expression of both Cx26 and Cx30 in the inner ear. He suggested that connexin 30 formed homotypic channels in the cochlea, but not sufficiently enough to prevent the loss of hearing. Therefore Cx30 does not provide a substitute to the loss of functioning Cx26 (Engel-Yeger *et al.*, 2002).

Further research concerning Cx30, a β-class connexin, revealed that the *GJB6* gene maps to chromosome 13q12, roughly 35 kb from *GJB2* (Figure 2.7). The *GJB6* gene encodes a protein that is 261 amino acids in length and is the fourth connexin linked to hearing loss (Grifa *et al.*, 1999; Pallares-Ruiz *et al.*, 2002). The Cx30 protein shares 77% homology with the Cx26 protein, but has 35 additional amino acids at its C-terminus (Kelley *et al.*, 1999).

Figure 2.7: Diagram of* GJB6 *(Cx30) in relation to* GJB2 *(Cx26) and* GJA3 *(Cx46)



Adapted from Pallares-Ruiz *et al.*, 2002.

2.6.1 THE FUNCTIONS OF CONNEXIN 30

Research conducted in 1996 by Dahl *et al.* found that mouse Cx30 was expressed predominantly in the brain and to a lesser degree in the eyes, skin, lungs, testis and uterus. In later studies it was reported that human Cx26 and Cx30 were both expressed in the same cells of the cochlea. Specifically they were co-expressed in the spiral limbus, spiral ligament, basal region of the stria vascularis, and between the supporting cells of the organ of Corti (Kelley *et al.*, 1999). It was also noted that because Cx26 and Cx30 are structurally highly related (Figure 2.8), they could be co-expressed in the inner ear forming homo- and/or hetero-channels. It was thought that this expression may be a prerequisite for the maturation of the cochlea (Pallares-Ruiz *et al.*, 2002).

Figure 2.8: Comparison of the amino acid sequence of Cx26 and Cx30

Connexin	Amino Acid Sequence
Cx26	MDWGTLQTILGGVNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGD
Cx30	MDWGTLHTFIGGVNKHSTSIGKVWITVIFIFRVMILVVAAQEVWGD
Cx26	EQADFVCNTLQPGCKNVCYDHYFPISHIRLWALQLIFVSTPALLVA
Cx30	EQEDFVCNTLQPGCKNVCYDHFFPVSHIRLWALQLIFVSTPALLVA
Cx26	MHVAYRRHEKKRKFIKGEIKSEFKDIEEIKTQKVRIEGSLWWTYT
Cx30	MHVAYYRHETTRKFRRGEKRNDFKDIEDIKKHKVRIEGSLWWTYT
Cx26	SSIFFRVIFEAAFMYVFYVMYDGFSMQRLVKCNAWPCPNTVDCFVS
Cx30	SSIFFRIIFEAAFMYVFYFLYNGYHLPWVLKCGIDPCPNLVDCFIS
Cx26	RPTEKTVFTVFMIAVSGICILLNVTELCYLLIRYCSGKSKPV- - - -
Cx30	RPTEKTVFTIFMISASVICMLLNVAELCYLLLKVCFRRSKRAQTQK

Cx26	- -
Cx30	N H P N H A L K E S K Q N E M N E L I D S G Q N A I T G F P S

Variations in amino acid sequence are indicated by red text (X) and the absence of an amino acid is indicated by a red dash (-). Adapted from the sequences deposited in the NCBI with accession number NM_004004 for Cx26 and NM_006783 for Cx30.

2.6.2 MUTATIONS OF *GJB6* THAT ARE INVOLVED IN DFNB1 HEARING IMPAIRMENT

To date, only two mutations in *GJB6* (OMIM: 604418) linked to autosomal dominant deafness have been identified together with six polymorphisms (Table 2.6). The number of mutations in *GJB6* that cause non-syndromic autosomal recessive hearing loss is relatively low (Kelley *et al*., 1999). The only mutation in *GJB6* that has been linked to recessive non-syndromic deafness is a large deletion, known as Δ(*GJB6*-D13S1830) that involves the loss of approximately 342 kb of DNA (Connexin Homepage, 2003), which is discussed in detail in Section 2.6.2.1.

Table 2.6: Sequence variants that have been identified in* GJB6 *in autosomal dominant deafness

Mutations that cause non-syndromic autosomal dominant deafness			
Mutation name	**Description**	**Effect**	**Protein domain**
T5M	C to T at 14	Threonine at 5 into Methionine	NT
63delG	deletion of G at 63	Frameshift	E1
Variants of unknown effect (polymorphisms)			
Polymorphisms	**Description**	**Frequency**	**Protein domain**
A39A	T to C at 117	not described	M1
P58P	G to A at 174	not described	E1
L132L	G to A at 396	not described	M3
S139G	A to G at 415	not described	M3
L163L	G to A at 489	not described	E2
S199T	T to A at 595	not described	M4

Adapted from the connexin homepage, 2003.

The *GJB6* gene has also been implicated in causing HED, which is also referred to as Clouston syndrome. This syndrome is an autosomal dominant skin disorder that is characterized by palmoplantar hyperkeratosis, nail hypoplasia and deformities, as well as hair defects. *GJB6* is also involved in

autosomal dominant non-syndromic hearing loss (Lamartine *et al.*, 2000). This again is an example of how different mutations found within connexins result in various disorders. Another case is the mutations found in the *GJB3* gene that encodes connexin 31 (Cx31). Mutations in this gene are responsible for autosomal dominant deafness as well as for the skin disorder erythrokeratodemia variabilis (EKV, OMIM: 133200 and 605425.001). From these findings it could be assumed that different mutations in the connexin genes can result in either skin disorders or deafness depending on where the mutations are located within the gene (Maestrini *et al.*, 1999).

*2.6.2.1 THE Δ(*GJB6-*D13S1830) MUTATION*

A deletion of 342 kb, known as Δ(*GJB6*-D13S1830), was first identified in the Spanish population. This deletion dissects the *GJB6* gene but does not affect the *GJB2* gene and is usually observed together with mutations found in *GJB2* (Del Castillo *et al.*, 2002). A second deletion of approximately 140 kb was identified in the Ashkenazi Jewish population as well as in the French population (Lerer *et al.*, 2001; Pallares-Ruiz *et al.*, 2002). Again, it was noted that these deletions dissected *GJB6* but did not affect *GJB2* (Lerer *et al.*, 2001; Pallares-Ruiz *et al.*, 2002). In the past there was confusion as to whether these deletions were distinctly different but it has been shown in a recent study that all the patients in the previous studies contained the same deletion breakpoint junction, thereby confirming that all the cases were those of the Δ(*GJB6*-D13S1830) mutation. Using the latest sequencing information it has also been established the Δ(*GJB6*-D13S1830) mutation is 309 kb long and not the previously estimated size of 342 kb (Del Castillo *et al.*, 2003).

Within the Spanish population the Δ(*GJB6*-D13S1830) deletion is the second most frequent mutation causing non-syndromic autosomal recessive deafness, after 35delG, in *GJB2* (Del Castillo *et al.*, 2002). The frequency of this deletion has also been determined in a number of other populations as is shown in Table 2.7. It must be noted that there is a relatively low number of Δ(*GJB6*-D13S1830) homozygous cases. This is the result of the incidence rate of this mutation being too low to cause a high frequency of patients with the homozygous genotype to be present in any of the populations studied (Del Castillo *et al.*, 2003).

Table 2.7: Allele frequency of Δ(GJB6-D13S1830) within several populations.

Popoulation	Allele Frequency (%)[a]
Spain (Madrid)	7.6
Spain (Barcelona)	9.7
Italy	0.0
France	8.2
Belgium	1.4
United Kingdom	5.9
Israel (Tel Aviv)	7.1
Israel (Jerusalem)	6.0
USA (Virginia)	4.5
Israel (Iowa)	1.6
Brazil	7.1
Australia	1.3

[a] Calculated from the number of Δ(*GJB6*-D13S1830) alleles identified divided by the total number of DFNB1 alleles, which only included those cases were both the DFNB1 alleles had been identified.
Adapted from Del Castillo *et al.*, 2003.

It was unclear as to whether the Δ(*GJB6*-D13S1830) mutation was due to a founder effect or the presence of a mutational hotspot in that area of the chromosome. The latest findings, involving haplotype analysis, have indicated that the mutation is the result of a founder effect especially amongst the Ashkenazi Jewish population. It also appears that there is a common ancestor responsible for this deletion amongst those populations found within those countries of Western Europe. The haplotypes that were obtained from the Ashkenazi patients with non-syndromic deafness showed a similarity along a 464 kb region. When this was compared with those that were obtained from Western Europe it was noted that there was more diversity amongst those individuals from Western Europe, suggesting that there was an older founder for this mutation as well as the possibility of various points of origin amongst these countries (Del Castillo *et al.*, 2003).

There are various ways in which this large deletion could result in non-syndromic autosomal recessive hearing impairment (Del Castillo *et al.*, 2002; Pallares-Ruiz *et al.*, 2002):

- A digenic mode of inheritance is the result of either the removal of two doses of *GJB6* or the removal of one dose of *GJB6* together with one dose of *GJB2*. This would lead to the reduction

in the number of homomeric or heteromeric connexons present in the inner ear and therefore there would be insufficient transportation of K ions.

- A monogenic mode of inheritance due to the removal of the regulatory elements that are essential for the expression of *GJB2* in the cochlea. This regulatory element would be found far upstream of the *GJB2* gene and the deletion would result in the suppression of the expression of Cx26. However, this regulatory element of *GJB2* is still hypothetical, as no such element has been identified.
- The resulting phenotype of hearing loss is probably due to a combination of both mechanisms.

2.7 OTHER CONNEXIN PROTEINS

Due to connexins being distributed in many organs and systems it has been observed that mutations in connexin genes affect these areas of the body causing different disorders. It has even been noted that the same connexin gene can be associated with different diseases. Mutations in connexins have been predominantly linked to alterations in the auditory system, peripheral nerves and the skin (Rabionet *et al.*, 2002).

Besides *GJB2* and *GJB6*, there are other connexin genes that are associated with hearing loss. Firstly, there is *GJB3* (Cx31) that has been linked to both non-syndromic autosomal recessive deafness as well as non-syndromic autosomal dominant deafness. The dominant form is characterized by bilateral high-frequency hearing impairment. Secondly, a missense mutation in *GJA2* (Cx43) that encodes an α-connexin has been reported as the cause of autosomal recessive deafness in several African-American families (Rabionet *et al.*, 2002).

From research conducted, it appears that gap junction communication plays an important part in the coordinated control of epidermal keratinocyte differentiation. It has also been reported that all known β-connexin genes are expressed in human skin cells. The first connexin gene to be reported to cause the autosomal dominant skin disorder EKV is *GJB3*. All mutations in this gene are missense changes that affect several domains of the protein and lead to variable clinical symptoms. A second gene *GJB4*

(Cx30.3) has also been found to co-segregate with EKV, associated with erythem gyratum repens (Rabionet *et al.*, 2002).

There are several types of Charcot-Marie-Tooth (CMT, OMIM: 302800) an inherited peripheral neuropathy caused by different genes. Two connexin genes, *GJB1* (Cx32) and *GJB3*, have been linked to this disorder. Mutations in *GJB1* lead to the X-linked form that has a pattern of inheritance that is both dominant and recessive, where males have a more severe phenotype than their carrier female counterparts who show a milder form of the disease. This disorder has characteristic features of progressive atrophy of distal muscles, a decreased number of myelinated fibers as well as the formation of onion bulbs, which consist of supernumerary, flattened Schwann cells. To date, more than 240 mutations have been identified in *GJB1* since the gene was discovered in 1993. Only recently a dominant mutation in *GJB3* was reported to cause a rare type of peripheral neuropathy that is characterized by a wide range of disease severity. These features can range from asymptomatic cases or individuals with persistent skin ulcers on their feet and osteomyelitis, which leads to amputations. Symptoms of mild, asymmetrical hearing loss have also been reported together with the neuropathy in some patients (Krutovskikh and Yamasaki, 2000; Rabionet *et al.*, 2002).

Finally, connexin genes have also been reported to play a role in the formation of cataracts. Specifically, mutations in *GJA3* have been linked to autosomal dominant congenital cataracts and Cx50 appears to play a role in human "zonular pulverulent" cataracts (Krutovskikh and Yamasaki, 2000).

2.8 GENETIC COUNSELLING FOR NON-SYNDROMIC AUTOSOMAL RECESSIVE HEARING IMPAIRMENT

Autosomal recessive non-syndromic deafness is relatively common with a frequency of 1 in 1 000. This rate can be compared with other common disorders such as Down syndrome (1 in 600 - 800), cleft lip and palate (1 in 750) and cystic fibrosis (1 in 3 500 Caucasians or 1 in 17 000 African-Americans) in the United States (Kenna *et al.*, 2001). Due to the high frequency of hearing loss, there has been an increasing demand for a fast and precise screening service for the detection of the various mutations in the *GJB2* gene. This service needs to be population based so that it can provide the most accurate results possible in the shortest amount of time with the least expenses being incurred (Rickard *et al.*,

2001; Najmabadi *et al.*, 2002). The screening of *GJB2* for mutations was the first genetic test clinically available for autosomal recessive non-syndromic hearing impairment (Lench *et al.*, 1998).

There are a number of reasons why many families want to know why their children are suffering from hearing impairment. This includes how it will affect their educational plans for the child, the method of communication chosen, what type of hearing device or other aid should be considered, as well as whether a cochlear transplant is a viable option. Another important reason for genetic counselling is to determine whether there is a chance of having further children with hearing loss (Kenna *et al.*, 2001). According to current information it has been estimated that the chance of a normal-hearing couple having a second child with hearing loss is between 15 and 20%. If the diagnosis determines that the cause of deafness was not due to *GJB2* mutations this risk drops to 14%, whereas if it was determined that *GJB2* was the cause the risk factor is raised to 25% (Green *et al.*, 1999; Prasad *et al.*, 2000).

The manner in which people determine if a condition is serious depends on their culture, socio-economic status, religion and personal experience. Therefore, these factors differ amongst populations as well as communities (Pampanos *et al.*, 2002). In the case of the deaf community there as some individuals that do not consider that having a hearing impairment should be considered a serious medical condition whereas the majority of those outside the community see it as a major handicap. Therefore, the challenge to those that are providing a genetic counselling service is that they must be sympathetic and sensitive to all points of view (Wilcox *et al.*, 1999).

2.8.1 EVALUATION OF HEARING IMPAIRMENT

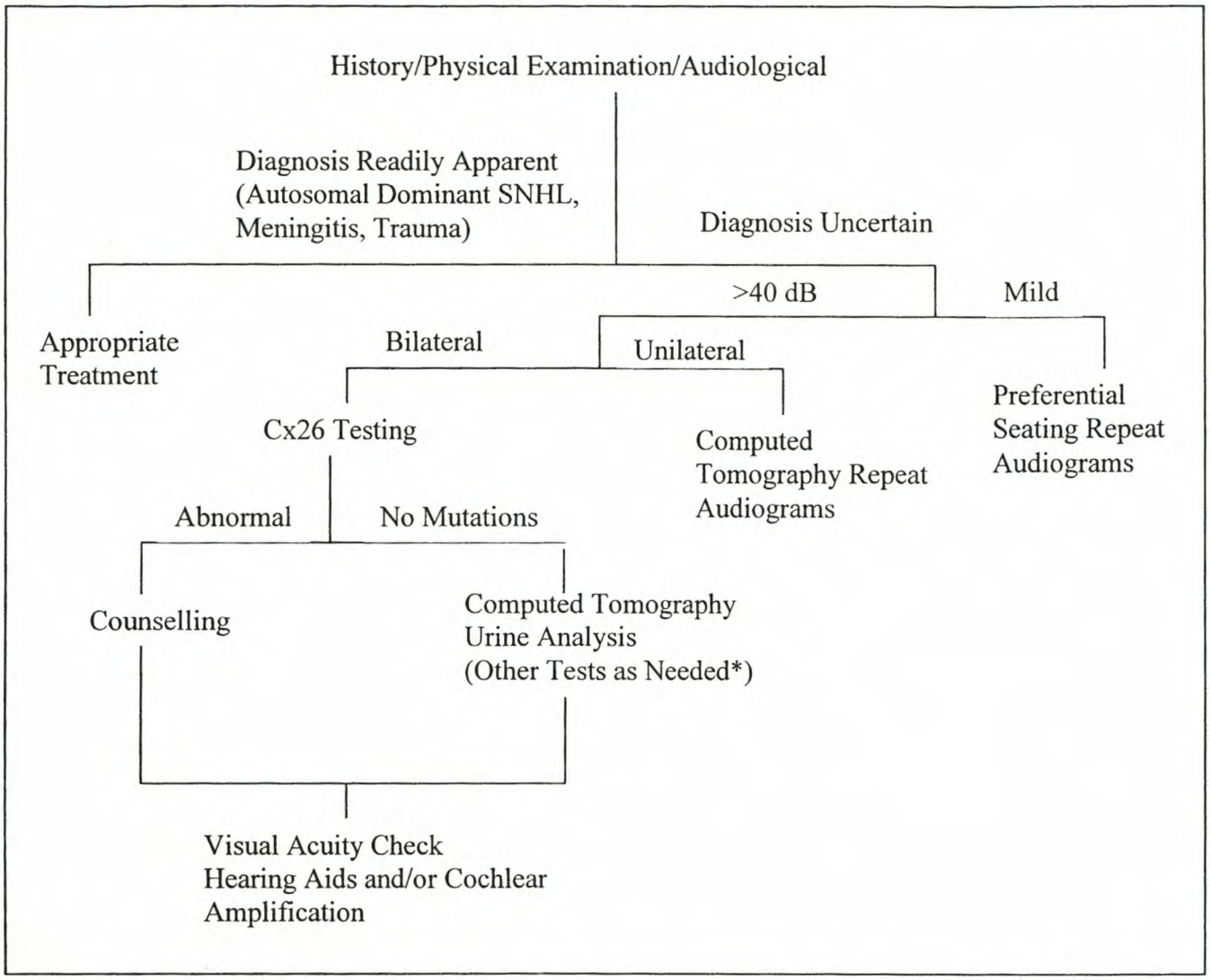
The standard tests that are conducted to determine the cause of deafness when a child is newly diagnosed includes thyroid, renal, liver and immunologic function tests as well as assessments for syphilis, toxoplasmosis and cytomegalovirus. The child can also be referred to ophthalmology and neurology specialists. Recently advances in medicine have lead to more accurate diagnostic techniques being developed for the use in hearing impairment identification. These new techniques include high-resolution topography and magnetic resonance imaging (MRI) of the temporal bone (Kenna *et al.*, 2001).

Previously, genetic testing was limited to dysmorphologic examinations together with a detailed evaluation of the families' medical history. These inaccurate methods that were used in the past are now making way for more accurate assessments based on the identification of the genes that are involved in syndromic or non-syndromic deafness. This has lead to genetic counseling becoming an important tool in the early diagnosis of hearing loss. (Kenna *et al.*, 2001).

There is some debate as to where in the evaluation procedure the genetic testing for *GJB2* mutations should be placed as well as to who should be considered for such testing. On the one hand it has been suggested that it should be the first test to be conducted, as all further tests would be unnecessary if a positive result for *GJB2* mutations is obtained, thereby saving time as well as resources. Conversely, it has been suggested that genetic analysis of *GJB2* should just be one of a number of tests conducted thereby ensuring accurate results, i.e. preventing false positives or negatives (Figure 2.9).

When it comes to determining whom should benefit from the investigation of the *GJB2* gene for mutations, it has been proposed that only those children who show the specific phenotypic features of DFNB1 should undergo testing. The other possibility is that all children showing possible non-syndromic hearing loss should be tested as deafness due to *GJB2* mutations has a high frequency amongst children and there is also such a variation in phenotypes that it is possible to incorrectly classify a case (Denoyelle *et al.*, 1999; Kenna *et al.*, 2001).

Figure 2.9: Diagnostic algorithm for sensorineural hearing loss (SNHL) in children.



* Indicates other tests: thyroid function; fluorescent treponemal antibody; erythrocyte sedimentation rate and/or Western blot analysis; platelet analysis; Pendred syndrome and bronchio-oto-renal syndrome; genetic studies; and electrocardiogram.

Adapted from Greinwald and Hartnick, 2002.

2.8.2 AVAILABLE TREATMENT FOR HEARING LOSS

The possible steps that can be taken for the treatment of hearing impairment include early speech and language developmental programs as well as the use of listening devices such as hearing aids and FM systems. Recent progress in technology has lead to the development of digital and programmable hearing aids together with improvements being made in the analog hearing aids (Kenna *et al.*, 2001).

The level of deafness in those suffering from autosomal recessive non-syndromic deafness due to mutations in the *GJB2* gene, often prevents the successful use of hearing devices. The option of a cochlear transplant is considered as a viable alternative. However, for this method to be successful there has to be surviving as well as electrically sensitive spiral ganglion cells and cochlear nerves. Both of these are present in patients with *GJB2* related deafness and cochlear implantation is now a regular alternative for those children that suffer from bilateral severe or profound deafness who would not sufficiently benefit from the use of another hearing device (Jun *et al.*, 2000; Kenna *et al.*, 2001). Further evidence supporting the use of cochlear transplants for *GJB2* non-syndromic autosomal recessive hearing loss has been reported by Fukushima *et al* (2002). He conducted a study that focused on the performance of patients with *GJB2* deafness compared to those patients with non-*GJB2* deafness who had received an implant. It was discovered that those with mutations in *GJB2* had better language development and cognitive ability after the transplant compared with those that were deaf due to other causes.

2.8.3 WHOLE GENE VERSUS 35delG SCREENING

There is some debate as to whether the whole *GJB2* gene or just the 35delG mutation should be screened. There are a number of reasons supporting and disputing both suggestions. The high frequency of the 35delG mutation of *GJB2* supports the idea of only screening for this mutation as complete results will be obtained in this manner for a number of individuals suffering from autosomal recessive non-syndromic hearing impairment (Greinwald and Hartnick, 2002). The 35delG mutation is also easy to detect as a number of methods have been developed recently for this purpose and it would be practical because it saves both time as well as resources (Denoyelle *et al.*, 1999).

However, in some populations only between 30 and 60% of children with non-syndromic autosomal recessive hearing loss are homozygous for the 35delG mutation found in the *GJB2* gene. Therefore, in these cases it would be necessary to screen the entire *GJB2* gene for other contributing mutations that could be found either in the coding, non-coding or promoter regions of *GJB2* (Denoyelle *et al.*, 1999; Wilcox *et al.*, 2000). It is possible to use direct DNA sequencing as a method to detect other possible mutations other than 35delG, since the *GJB2* gene is relatively small in size. This is the preferred technique as it is more accurate than other methods (Simsek *et al.*, 2001).

2.8.4 ADVANTAGES AND DISADVANTAGES OF CONDUCTING GENETIC TESTS FOR AUTOSOMAL RECESSIVE NON-SYNDROMIC DEAFNESS

The advantages of genetic screening of *GJB2* in children with autosomal recessive non-syndromic hearing loss are numerous. Below is a summary of a few of the most important reasons that have been provided for conducting such testing (Marlin *et al.*, 2001; Greinwald and Hartnick, 2002; Kemperman *et al.*, 2002):

- Genetic testing of children who are deaf would increase the number of those that are accurately diagnosed for autosomal recessive non-syndromic deafness. Previously they would have remained undiagnosed, due to there being no method for this type of hearing impairment. Currently between 25 and 52% of patients have undiagnosed non-syndromic hearing loss.
- Target testing for mutations that are population specific, such as 35delG, allows for a quick and accurate diagnosis that may prevent the need for further testing.
- Genetic analysis would also greatly help in identifying the cause of deafness in cases where there is no clear family history, in other words sporadic cases of autosomal recessive non-syndromic hearing loss. It has been estimated that a third of these cases would be explained.
- Genetic testing for *GJB2* mutations provides an opportunity for the families of affected children to get the appropriate genetic counseling that would provide them with the correct information, and which would help in making suitable decisions about treatment and education. Early diagnosis leads to treatment being attained earlier, which provides better results in the long term especially in the case of cochlear transplants.
- Genetic counselling would be able to provide important information concerning the risks of future offspring who may also suffer from autosomal recessive non-syndromic deafness.

A number of disadvantages have also been suggested concerning the genetic analysis of the *GJB2* gene in those suffering from autosomal recessive non-syndromic hearing impairment. The following list is a summary of the most important points that have been raised concerning such screening (Green *et al.*, 2000; Prasad *et al.*, 2000; Greinwald and Hartnick, 2002):

- ❖ High rates of false-positives and false-negatives have been recorded. In the case of the detection of the 35delG mutation a frequency of 2.6 and 3.1% respectively has been recorded. This is mainly due to the screening methods used, therefore the results can be improved by using automated DNA sequencing.
- ❖ The unclear relationship between genotype and phenotype can produce misleading results that may lead to incorrect genetic counselling.
- ❖ It is still difficult to determine whether certain missense mutations do in fact result in deafness or if the variation in the DNA sequence is just a benign polymorphism.
- ❖ A major ethical issue that has been raised concerns the possibility of parents wanting to terminate a pregnancy due to prenatal genetic tests indicating the likelihood of having a child who will suffer from autosomal recessive non-syndromic hearing loss. The final decision will have to take into account that there is a large variation in the degree of hearing loss that is experienced by those suffering from deafness related to *GJB2* mutations and that this variation even occurs between family members.

2.9 METHODS USED FOR MUTATION DETECTION

The procedures used for the detection of mutations can be divided into two steps: firstly, the identification of changes in the primary DNA structure by the variation of the physical or enzymatic properties due to the presence of a mutation, and secondly, the visualization of this change. The different methods used in mutation analysis (Table 2.7) can be divided according to whether they are founded on the presence of prior knowledge of the characteristics of the mutation or whether the methods are used to screen for unknown mutations (Kristensen *et al.*, 2001).

Table 2.7: Mutation detection techniques based on (A) prior knowledge of the variant or (B) information available

(A) Analysis of known variants	**(B) Screening for unknown variants**
Restriction fragment length polymorphism (RFLP)	Single-strand conformation polymorphism (SSCP) and heteroduplex analysis (HA)
Allele specific oligonucleotide analysis (ASO-PCR)	DNA sequencing
Invasive cleavage of nucleotide probes	Random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR (AT-PCR)
Ligation-based assays	Direct termination PCR (DT-PCR)
Double-strand conformation analysis (DSCA)	Amplified fragment length polymorphism (AFLP)
Molecular beacons	Cleavage fragment length polymorphism (CFLP), enzymatic mutation detection (EMD)
Microarrays "loss of signal"	Microarrays "gain of signal"
Fluorescence resonance energy transfer (FRET)	Minisequencing

Adapted from Kristensen *et al.*, 2001.

A number of different techniques have been used to detect the various mutations found within the *GJB2* gene and specifically the 35delG mutation. These methods include amplification-resistant mutation screening (ARMS), allele specific oligonucleotide analysis (ASO), allele-specific polymerase chain reaction (PCR) and PCR-mediated site-directed mutagenesis. All these methods that have been used have some or other disadvantage, be it expense, time or limited sensitivity (Smith and Van Camp, 1999; Antoniadi *et al.*, 2000; Lin *et al.*, 2001).

One of the techniques that has been relatively successful in the detection of mutations in *GJB2* is the single-stranded conformation polymorphism (SSCP) method, which involves the electrophoretic discrimination of allelic variants (Kelley *et al.*, 1998; Carrasquillo *et al.*, 1997). The standard process of SSCP analysis entails the denaturing of PCR-amplified fragments into single strands followed by the formation of sequence-specific secondary and tertiary structures that are produced in a non-denaturing solution during gel electrophoresis. The three-dimensional structure that forms is dependent on the nucleotide sequence and determines the mobility of the molecule. Those strands that differ even by a single base have distinct conformations that can be visualized by the variation in migration pattern. The advantages of SSCP analysis include that it is simple, relatively inexpensive and highly sensitive (80%) for fragments up to 300 bp (Hayashi and Yandell, 1993). However, this technique does have its disadvantages, which include that the sensitivity decreases with an increase in fragment length,

therefore the sizes of the fragments screened are limited. There is a low detection rate of G to C transitions (Orita *et al.*, 1989; Ganguly *et al.*, 1993; Kristensen *et al.*, 2001; Sambrook and Russell, 2001).

Automated DNA sequencing is considered the ultimate mutation detection method that can be used to identify both known and unknown changes in the DNA arrangement as it is the most accurate technique developed to date (Kristensen *et al.*, 2001). This technique involves the use of four different dyes as labels in base-specific reactions. The labels can either be connected to base-specific ddNTPs or to the primer, in which case there have to be four sets of primers to correspond to the four different reactions. A laser beam is focused on a specific point on the gel during electrophoresis so that as the DNA fragments migrate past this point, the laser can pick up the specific wavelengths of the different fluorescent dyes. The information that is generated is then stored electronically in a computer database (Strachan and Read, 1999). This is the ideal method to use in the screening of *GJB2* mutations, because the *GJB2* gene is small. It is therefore a relatively fast procedure because the entire coding region can be analyzed in a single reaction. Another advantage of using sequencing as a screening tool is that it can detect all mutations present, even those that are rare (Green *et al.*, 1999; Wilcox *et al.*, 2000; Kemperman *et al.*, 2002). The main disadvantage of this technique is that it is not easily applicable in routine diagnostic laboratories because it is expensive and time consuming (Storm *et al.*, 1999; Antoniadi *et al.*, 2000).

The techniques that have been used successfully with regards to the detection of mutations within *GJB6* that play a role in non-syndromic autosomal recessive deafness include SSCP and hetroduplex analysis. These techniques were used together with direct automated DNA sequencing to identify the specific variants that were produced (Grifa *et al.*, 1999; Kelley *et al.*, 1999 and Gabriel *et al.*, 2001). These same techniques have also been used successfully for the identification of mutations that result in non-syndromic autosomal recessive hearing loss involving other connexin genes, such as *GJB3*, *GJA1* and *GJB4* (Liu *et al.*, 2000 and 2001; López-Bigas *et al.*, 2000, 2001 and 2002)

2.10 OBJECTIVES OF THIS STUDY

The identification of the *GJB2* and *GJB6* mutations in the South African population that underlie autosomal recessive non-syndromic deafness will lead to the broadening of our understanding of the biological role of the *GJB2* and *GJB6* gene mutations. This information would also provide an answer for families and individuals as to the cause of their deafness and improve the genetic counselling that is offered to families and individuals at risk of developing non-syndromic hereditary hearing impairment.

The specific aims of this study were:

1. The investigation of the entire coding region of *GJB2* to identify any mutations that result in non-syndromic autosomal recessive deafness in the Caucasian and Mixed Ancestry populations of South Africa.
2. The investigation of the entire coding region of *GJB6* to identify any mutations that result in non-syndromic autosomal recessive deafness in the Caucasian and Mixed Ancestry populations of South Africa.
3. The application of three SSCP gel electrophoresis systems to determine which was best suited for use in mutation screening of *GJB2* in a standardised diagnostic program. The three systems that were tested included a mini PAGE procedure, a SSCP-urea procedure and a two buffer system.
4. The determination of the frequencies of mutations detected in *GJB2* and *GJB6* amongst the Caucasian and Mixed Ancestry populations of South Africa. This information indicates which mutations are common to these populations, therefore allowing for a population specific diagnostic program to be put in place in the future.

CHAPTER 3: MATERIAL AND METHODS

The Ethics Review Committee of the Faculty of Health Sciences, Stellenbosch University approved the study presented in this thesis (Project number 99/125). Prior to the collection of blood samples, written informed consent was obtained from all individuals included in this study.

3.1 SAMPLE COLLECTION

The families studied in this project were referred mainly from the Carl du Toit Centre, the De La Bat School and Aftercare Centre as well as the Nuwe Hoop Centre situated in the Western Cape. However, three families were recruited from the Bartemeu School in the Free State and one family from the Wits Clinic in Gauteng. For families to be entered into the project they had to provide written informed consent (Appendix A), and both the parents and children had to undergo audiological tests. In the case of the familial group there preferably had to be two unaffected parents and two affected children present.

Mrs Muller (Division of Audiology, Tygerberg Hospital) conducted the clinical evaluations, which included the audiology tests. The pedigree analysis and prenatal history of the affected children was conducted by Dr. G de Jong of the Division of Human Genetics, University of Stellenbosch, Tygerberg. Using the same specialist for all the clinical examinations prevented the possibility of diagnostic bias. The individuals who participated in this study received genetic counseling as well as information regarding the results of the molecular screening.

A total of 44 families were recruited, and 125 individuals were screened for mutations in the *GJB2* and *GJB6* genes. These families were divided into two groups, either familial or sporadic, according to the definition that a case was familial if the condition was found in a number of members of a family whereas sporadic cases were those that occurred only in isolated individuals (Oxford Concise Medical Dictionary, 2000). The familial group comprised 16 families and the sporadic group was made up of 28 families. The familial group predominantly contained Caucasian families except for families 12 and 13 who were of Mixed Ancestry (consisting of Khoi San, Malay, African and European Caucasian ancestry). Again, the sporadic group was also primarily Caucasian except for families 9, 18, 22 and 28

who were of Mixed Ancestry; families 14, 15 and 16 who were African; and families 10, 11 and 21 who were of Indian origin.

The age of the affected individuals who were screened for *GJB2* and *GJB6* mutations varied from four months to 44 years, however, all were diagnosed with hearing impairment before the age of two years. All those affected by non-syndromic hearing loss had hearing difficulty ranging from severe to profound deafness.

Control samples obtained from non-related individuals who appeared to have normal hearing were also screened in this study. In total, 50 Caucasian and 50 Mixed Ancestry control samples were screened for mutations in both *GJB2* and *GJB6* genes.

3.2 DNA EXTRACTION

DNA was extracted from 5-10 ml whole blood samples that had been collected in tubes containing ethylendediamine tetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$) as the preservative. The method used was a modification of the one described in 1998 by Miller *et al.* This method involved adding 30 ml cold lysis buffer [155 mM ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate ($KHCO_3$), 0.1 mM EDTA – pH 7.4) to each whole blood sample that had been transferred to 40 ml centrifugation tubes [Greiner Labortechnik, Kremsmuenster, Austria]. This solution was kept on ice for 15 minutes until the red blood cells had undergone lysis, then the cell suspension was centrifuged at 201 x g for 10 minutes at 4°C [Beckman Avanti 30™, Beckman Coulter, Inc, Fullerton, CA, USA]. The supernatant was removed and the pellet was resuspended in 10 ml cold phosphate buffered saline solution (PBS) [6 mg/ml potassium chloride (KCl), 24 mg/ml sodium chloride (NaCl), 3.42 mg/ml di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 0.6 mg/ml potassium dihydrogen orthophosphate (KH_2PO_4)]. This solution was centrifuged at 201 x g for 10 minutes at 4°C and the supernatant was once again removed. The intact pellet was resuspended in 3 ml nucleic lysis buffer [10 mM Tris(hydroxymethyl)aminomethane (Tris-HCl) ($(CH_2OH)_3CNH_2$-Cl), 400 mM NaCl, 2 mM EDTA – pH 8.2] together with 150 µl 20% (w/v) sodium dodecyl sulphate (SDS) and 30 µl proteinase K (10 mg/ml) [Roche Diagnostics Corporation, Indianapolis, IN, USA]. This solution was incubated overnight in a water bath at 50°C.

Following after overnight incubation, 1 ml of saturated 6 M NaCl was added and the solution was shaken vigorously for one minute, followed by centrifugation at 559 x g, at 21°C for 15 minutes. The supernatant containing the DNA was transferred to a clean 40 ml centrifugation tube, with care taken to leave the foam and pellet behind. The supernatant was then centrifuged at 559 x g, at 21°C for 15 minutes. The supernatant was transferred to a 30 ml glass Porex tube [QSP® (Quality Scientific Plastics) Porex Bio Products Group, Fairburn, GA, USA] and the volume was noted. To this, two times the volume of ice-cold 100% (v/v) ethanol (EtOH) was added and the solution was covered with Parafilm® [American National Can™, Menasha, WI, USA] and left at room temperature for 30 min to allow the DNA to precipitate. The precipitated DNA was transferred, to a clean 1.5 ml Safe Lock Eppendorf® tube [Eppendorf, Hamburg, Germany] with an innoculation needle [Looplast®, LP Italiana, SPA, Milan, Italy]. The DNA was washed with 300 µl ice-cold 70% (v/v) EtOH to remove any excess salt. This solution was centrifuged at 17 530 x g for 15 minutes at 4°C [Beckman GS-15R, Beckman Coulter, Inc, Fullerton, CA, USA] followed by the careful removal of the excess EtOH. The DNA was left to air dry at room temperature. This DNA pellet was dissolved in 250 µl double distilled (ddH_20) SABAX water [Adcock Ingram, Johannesburg, RSA] overnight at room temperature after which the DNA was stored at 4°C until further use.

3.2.1 DETERMINATION OF EXTRACTED DNA CONCENTRATION

To determine the concentration of the genomic DNA (gDNA) that was extracted from the whole blood samples a concentration gel was resolved. This involved the loading of the extracted gDNA samples onto a horizontal 2% (w/v) agarose gel. 5 µl of gDNA was mixed to an equal volume of 6X loading dye [0.25% (w/v) bromophenol blue ($C_{14}H_{10}BrO_5S$), 0.25% (w/v) xylene cyanol FF ($C_{25}H_{27}N_2Na_4O_6S_2Na$) and 30% (w/v) glycerol ($C_3H_8O_3$)]. A concentration gradient was made up of lambda (λ) DNA [Promega Corporation, Madison, WI, USA] of varying concentrations, ranging from 10 - 70 ng. The 2% (w/v) agarose gel was resolved in 1X TBE buffer [10.8 mg/ml Tris, 5.5 mg/ml Boric acid (H_3BO_3) and 0.7445 mg/ml EDTA - pH 8.3] for two hours at 120 V after which it was visualized under ultraviolet (UV) light and photographed using the GelDoc system [BioRad, Hercules, CA, USA].

3.3 POLYMERASE CHAIN REACTION AMPLIFICATION

3.3.1 POLYMERASE CHAIN REACTION AMPLIFICATION OF *GJB2*

The coding region of the *GJB2* gene was first amplified by producing five overlapping fragments (fragments 1,2,4,5 and 6), which were used in SSCP analysis (Table 3.1 and Figure 3.1). The fragments were produced because SSCP analysis is most effective if smaller fragments are used. Fragment 3, which covers the entire coding region of *GJB2* was used for DNA sequencing.

Table 3.1: Primers used in amplifying the coding region of the* GJB2 *gene

Fragment Number	Fragment Size (bp)	Primer Name	Primer Sequence	T_m (°C)	Annealing Temperature (°C)
1	350	1A (F)	5'-TCT TTT CCA GAG CAA ACC GC-3'	60	60
		1B (R)	5'-GAC ACG AAG ATC AGC TGC AG-3'	62	
2	500	2A (F)	5'-CCA GGC TGC AAG AAC GTG TG-3'	64	60
		2B (R)	5'-GGG CAA TGC GTT AAA CTG GC-3'	62	
3	750	1A (F)	5'-TCT TTT CCA GAG CAA ACC GC-3'	60	60
		2B (R)	5'-GGG CAA TGC GTT AAA CTG GC-3'	62	
4	490	4A (F)	5'-AGG CCG ACT TTG TCT GCA ACA-3'	64	66
		4B (R)	5'-GTG GGC CGG GAC ACA AAG-3'	60	
5	110	5A (F)	5'-CGA AGC CGC CTT CAT GTA CG-3'	64	60
		4B (R)	5'-GTG GGC CGG GAC ACA AAG-3'	60	
6	520	4A (F)	5'-AGG CCG ACT TTG TCT GCA ACA-3'	64	66
		2B (R)	5'-GGG CAA TGC GTT AAA CTG GC-3'	62	

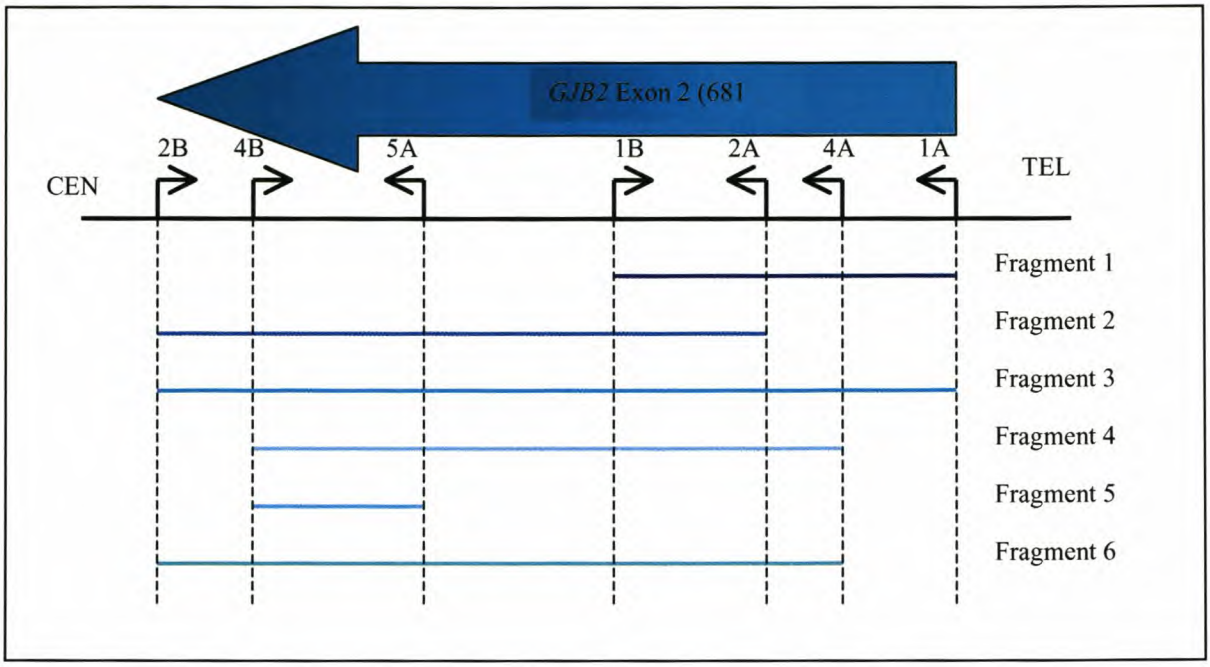
Primers synthesized by IDT® [Integrated DNA Technologies, Inc, Coralville, IA, USA].

The melting temperature (T_m) for each of the primers was calculated according to the equation described by Thein and Wallace (1986). The equation is as follows:

$$T_m = 2(A + T) + 4(G + C)$$

PCR conditions were optimized for each primer set with regard to the annealing temperature and the magnesium chloride ($MgCl_2$) concentrations.

Figure 3.1: Schematic representation of* GJB2 *indicating the position of the various primers



The PCR reactions were prepared in a total volume of 25 µl in 0.2 ml thin wall PCR tubes [QSP® (Quality Scientific Plastics) Porex Bio Products Group, Fairburn, GA, USA]. Before use the PCR reagents were thawed on ice and thoroughly mixed by vortexing. For fragments 2, 4, 5 and 6, the PCR was performed in reactions containing approximately 100 ng of gDNA that was extracted from the EDTA blood samples, 0.03 µM of the forward primer (Table 3.1), 0.03 µM of the reverse primer (Table 3.1), 50 µM of each 2'-deoxynucleotide (dNTP) [Promega Corporation, Madison, WI, USA], 0.25 U of *Taq* polymerase [BIOTAQ™ DNA Polymerase, Bioline, Springfield, NJ, USA] and a final concentration of 0.75 mM $MgCl_2$.

The standard PCR program that was used for fragments 2, 4, 5 and 6 began with denaturation at 95°C for five minutes. This was followed by 34 cycles consisting of denaturation at 95°C for one minute, annealing at the optimized temperature (Table 3.1) for two minutes and extension at 72°C for ten minutes. A final step of ten seconds at 25°C ensured that all PCR products were cooled to room temperature. All PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 [PE Applied Biosystems, Warrington WA, Great Britain].

In the case of fragment 1 the PCR was performed in reactions containing a total volume of 25 µl. Each reaction contained approximately 100 ng gDNA, 0.06 µM of the forward primer 1A, 0.06 µM of the

reverse primer 1B, 50 μM of each dNTP, 0.5 U of *Taq* polymerase and a final concentration of 0.75 mM $MgCl_2$.

The PCR program that was used for fragment 1 began with denaturation for two minutes at 95°C. This was followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 30 seconds. A final extension of five minutes at 72°C followed by a further ten seconds at 25°C ensured that all the PCR products were full length products.

For fragment 3, which covers the entire coding region of *GJB2*, the PCR reactions were prepared in a total volume of 25 μl. The PCR was performed in reactions containing approximately 100 ng gDNA, 0.03 μM of the forward primer 1A, 0.03 μM of the reverse primer 2B, 50 μM of each dNTP, 0.25 U of *Taq* polymerase and a final concentration of 0.75 mM $MgCl_2$.

The standard PCR program that was used for fragment 3 began with denaturation at 95°C for two minutes. This was followed by 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at the optimized temperature of 60°C for four seconds and extension at 72°C for 30 seconds. A final extension of 72°C for five minutes allowed for all PCR products to reach their full length. This was followed by ten seconds at 25°C to ensure that all the samples returned to room temperature.

3.3.2 POLYMERASE CHAIN REACTION AMPLIFICATION OF *GJB6*

The coding region of the gene that encodes Cx30 was amplified as a single fragment. Due to this region being so small, only 786 bp, it was not necessary to divide it into smaller fragments for successful automated DNA sequencing to occur. The primers that were used to accomplish the amplification were primers Cx30.1 and Cx30.8 (Table 3.2 and Figure 3.2) previously published by Del Castillo *et al.* (2002). PCR amplification of *GJB6* was only conducted in those individuals where either one or both mutant alleles for non-syndromic autosomal recessive deafness had not been identified during *GJB2* mutation analysis.

The primers GJB6-1R and BKR-1, indicated in Table 3.2 and Figure 3.2, which produced the Del fragment, were used to identify those samples that could possibly contain the Δ(*GJB6*-D13S1830)

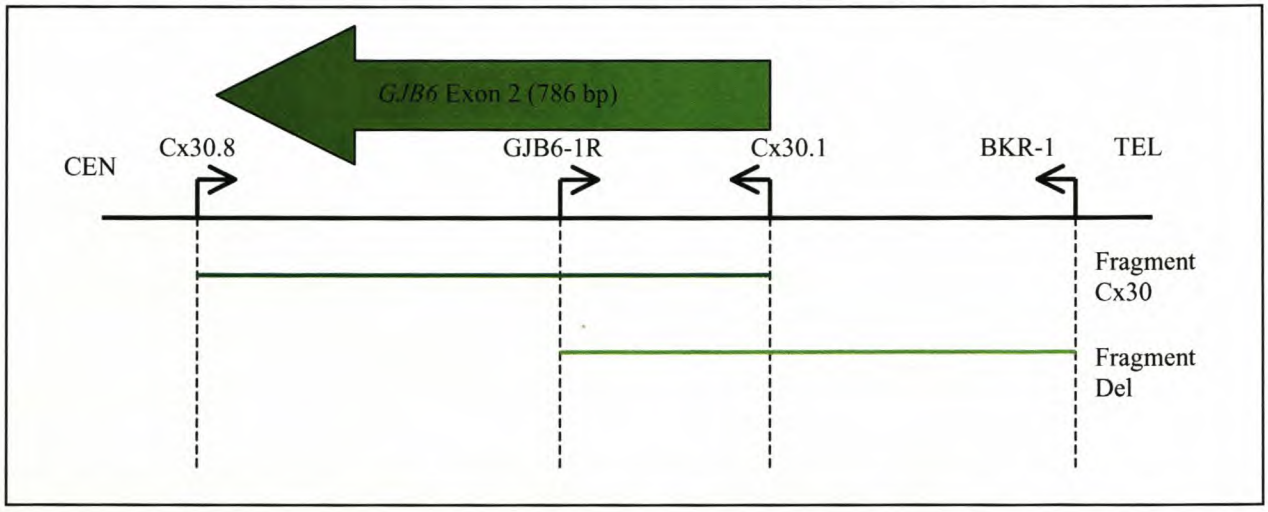
mutation that involves *GJB6* (Del Castillo *et al.*, 2002). PCR conditions were optimized with regard to the annealing temperature and the $MgCl_2$ concentration.

Table 3.2: Primers used in amplifying the coding region of the* GJB6 *gene

Fragment Name	Fragment Size (bp)	Primer Name	Primer Sequence	T_m (°C)	Annealing Temperature (°C)
Cx30	850	Cx30.1	5'-TCA GGG ATA AAC CAG CGC AAT-3'	58	56
		Cx30.8	5'-GTT GGT ATT GCC TTC TGG AGA AGA-3'	54	
Del	700	GJB6-1R	5'-TTT AGG GCA TGA TTG GGG TGA TTT-3'	68	50
		BKR-1	5'-CAC CAT GCG TAG CCT TAA CCA TTTT-3'	72	

Primers synthesized by IDT® (Integrated DNA Technologies, Inc, Coralville, IO, USA).

Figure 3.2: Schematic representation of* GJB6 *indicating the position of the various primers



Genomic DNA obtained from the extraction from the EDTA blood samples was used as template for amplification. The PCR reactions of fragment Cx30 were prepared in a total volume of 25 µl in 0.2 ml thin wall PCR tubes. Before use, the PCR reagents were thawed on ice and thoroughly mixed by vortexing. The PCR was performed in reactions containing approximately 100 ng of gDNA, 0.04 µM of the forward primer Cx30.1, 0.04 µM of the reverse primer Cx30.8, 50 µM of each dNTP, 0.25 U of *Taq* polymerase and a final concentration of 0.75 mM $MgCl_2$.

In the case of the PCR amplification of the Del fragment, the reactions were prepared in a total volume of 25 µl in 0.2 ml thin wall PCR tubes. Before use the reagents were thawed on ice and thoroughly

mixed by vortexing. The PCR was performed in reactions containing approximately 100 ng of gDNA, 0.6 μM of the forward primer GJB6-1R, 0.6 μM of the reverse primer BKR-1, 50 μM of each dNTP, 0.25 U of *Taq* polymerase and a final concentration of 2 mM $MgCl_2$.

The standard PCR program that was used for the fragments Cx30 and Del started with denaturation at 95°C for two minutes. This was followed by 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at the optimized temperature (Table 3.2) for 45 seconds and extension at 72°C for 30 seconds. A final extension of 72°C for five minutes followed by ten seconds at 25°C ensured that all PCR products were at their full length and the samples were returned to room temperature.

3.3.3 AGAROSE GEL ELECTROPHORESIS OF PCR AMPLICONS

After PCR amplification was completed the reactions were tested to determine if successful amplification had occurred. If this could not be done immediately the PCR products were stored at 4°C. Electrophoresis was performed using a horizontal 2% (w/v) agarose gel containing 0.01% (v/v) EtBr for staining purposes. An equal volume of 6X loading dye was mixed with 5 μl of PCR product. A 100 bp DNA ladder [Promega Corporation, Madison, WI, USA] was loaded on the gel, together with the PCR products, to be used as a molecular size marker. The 2% (w/v) agarose gel was resolved in 1X TBE buffer for one hour at 100 V after which it was visualized under UV light and photographed using the GelDoc system. Successfully amplified PCR products were stored at 4°C until further use.

3.4 COMPARISON OF GEL ELECTROPHORESIS SYSTEMS

At first it was decided to screen for mutations in *GJB2* using different SSCP gel electrophoresis systems to determine which is more effective at detecting all the different mutations found with in the coding region of the gene. The various procedures used included a mini polyacrylamide gel electrophoresis (PAGE) system, a 12% (w/v) polyacrylamide-urea SSCP system and finally, a two buffer system.

3.4.1 SINGLE-STRAND CONFORMATION POLYMORPHISM GEL ELECTROPHORESIS

3.4.1.1 MINI POLYACRYLAMIDE GEL ELECTROPHORESIS

The SSCP analysis using the mini polyacrylamide (PAA) gel electrophoresis system involved a 103 X 98 mm vertical gel with a composition of a 10% (w/v) PAA. The gel stock solution contained 5 ml of a 40% (w/v) acrylamide-bisacrylamide (19:1) mixture, 3 ml 5X TBE [54 mg/ml Tris, 27.5 mg/ml Boric acid and 3.7225 mg/ml EDTA - pH 8.3] and 2 ml dH_2O. A catalyst [60 µl TEMED ($C_6H_{16}N_2$)] and an oxidising agent [600 µl of a 10% (w/v) ammonium persulphate solution (APS)] were added to the gel to achieve polymerization of the 0.75 mm thick gel. Once polymerization had occurred the gel was placed in the gel apparatus [Mighty Small, Hoefer Pharmacia Biotech Inc, BioRad, Hercules, CA, USA] that contained fresh 1.5X TBE (16.2 mg/ml Tris, 8.25 mg/ml Boric acid and 1.116 mg/ml EDTA - pH 8.3) buffer in the buffer chamber. All the fragments except for fragment 6 were screened on this system.

The PCR products were prepared for electrophoresis by adding 10 µl of formamide loading buffer [98% (v/v) formamide (CH_3NO), 10 mM EDTA, 0.05% (w/v) xylene cyanol FF and 0.05% (w/v) bromophenol blue] to 20 µl of the PCR product. This solution was heat denatured for five minutes at 95°C and then placed on ice until loading. Approximately 10 µl of the denatured solution was loaded onto the gel.

Electrophoresis of the amplicons of fragment 1, 2 and 4 was performed at 200 V and 15°C for four hours, whereas for fragment 5 it was performed for two hours. After electrophoresis, the gels were dismantled and stained in 1X TBE containing 0.01% (v/v) EtBr for ten minutes and destained in dH_2O for approximately three minutes followed by visualization under UV light and photographed using the GelDoc system.

3.4.1.2 SSCP-UREA GEL ELECTROPHORESIS

In this SSCP system a 37 X 16.5 cm vertical gel with a composition of 12% (w/v) PAA, supplemented with urea [CH_4N_2O] was used. The gel stock solution contained 18 ml of a 40% (w/v) acrylamide-bisacrylamide (19:1) mixture, 4.5 g urea, 18 ml 5X TBE and 24 ml dH_2O. An oxidizing agent (600 µl

of 10% (w/v) APS) and a catalyst (60 µl TEMED) were added to the gel solution to achieve polymerization of the 0.75 mm gel. Once the gel had polymerized it was placed in the gel apparatus gel [Duel Slab Gel Unit, C.B.S. Scientific Co., Del Mar, CA, USA] that contained fresh 0.5X TBE buffer (5.4 mg/ml Tris, 2.75 mg/ml Boric acid and 0.37225 mg/ml EDTA - pH 8.3) in the lower chamber and fresh 1.5X TBE buffer in the upper chamber. All the fragments were screened using this gel electrophoresis system.

The amplicons of fragments 1, 2, 4 and 5 were prepared as before, by adding 10 µl formamide loading buffer to 20 µl PCR product. This solution was heat denatured for five minutes at 95°C and then placed on ice until 15 µl of the denatured solution was loaded onto the gel. In the case of fragment 6, it was first digested overnight with the restriction enzyme *MnlI* [Roche Diagnostics Corporation, Indianapolis, IN, USA (recognition sequence: 5'…CCT(N)$_{7/6}$…3')] before adding the loading buffer and heat denaturing. In each reaction 10 µl PCR product was utilized together with 2 µl Buffer 2, 0.2 µl bovine serum albumin (BSA) and 5 U *MnlI*. Finally, ddH_2O was added to a total volume of 20 µl in 1.5 ml Eppendorf® tubes and were incubated overnight in a 37°C water bath. After digestion, 20 µl of the digested fragment 6 together with10 µl loading buffer was heat denatured for five minutes at 95°C.

Electrophoresis of the amplicons of fragments 1, 2, 4 and 6 was performed at 300 V for approximately 18 hours at 4°C, whereas for fragment 5 it was performed for seven hours. Following electrophoresis, the gels were dismantled and stained in 1X TBE containing 0.01% (v/v) EtBr for ten minutes and destained in dH_2O for approximately three minutes after which the DNA fragments were visualized by UV light and photographed using the GelDoc system.

3.4.1.3 TWO BUFFER POLYACRYLAMIDE GEL ELECTROPHORESIS

The two buffer PAGE system used for this SSCP analysis involved producing a 37 X 16.5 cm vertical gel [Duel Slab Gel Unit, C.B.S. Scientific Co., Del Mar, CA, USA] with a 12% (w/v) composition of PAA supplemented with glycerol. The gel solution contained 12.5 ml of a 40% (w/v) acrylamide-bisacrylamide (19:1) mixture, 20 ml Tris-formate buffer [0.75 M Tris base and Formic acid, CH_2O_2 – pH 9.0] and 7 ml glycerol. To achieve polymerization of the 0.75 mm gel a catalyst (60 µl TEMED) and an oxidizing agent [600 µl of 10% (w/v) APS] were used. Once the gel had completely

polymerized it was placed into the gel apparatus that contained fresh Tris-formate buffer in the upper chamber and fresh Tris-borate buffer (125.9 g Tris base, 17.31 g Boric acid, 1 000 ml dH_2O – pH 9.0) in the lower chamber. Again, the PCR products of all the fragments were tested on this system.

The amplicons were prepared by adding 10 µl of formamide loading buffer to 20 µl PCR product. This solution was heat denatured for five minutes at 95°C and then placed onto ice until 10 µl of the denatured solution was loaded onto the gels. As before, fragment 6 was first digested with the restriction enzyme *MnlI* before the loading buffer was added and heat denaturing occurred. This involved adding 2 µl Buffer 2, 0.2 µl BSA and 5 U *MnlI* to 10 µl PCR product. The digestion solution was left overnight to incubate in a 37°C water bath. After digestion, 20 µl of digested fragment 6 together with 10 µl loading buffer was heat denatured for five minutes at 95°C.

Electrophoresis of the amplicons of all the fragments was performed at 350 V at 12 - 15°C for approximately two hours. Following electrophoresis, the gels were dismantled and stained in 1X TBE containing 0.01% (v/v) EtBr bromide for ten minutes and destained in dH_2O for approximately three minutes after which the DNA fragments were visualized by UV light and photographed using the GelDoc system.

3.5 AUTOMATED DNA SEQUENCING

The amplicons that were to be sequenced were purified using a QIAquick™ PCR Purification Kit [QIAGEN, GmbH, Hilden, Germany]. Fifty microliters Buffer PB was added to 10 µl PCR product. To bind the DNA, the sample was placed in a QIAquick™ spin column that was placed within a 2 ml collection tube. This was centrifuged for one minute at 10 000 x g. The flow-through was discarded and the spin column was placed back into the collection tube. To wash the DNA, 0.75 ml Buffer PE was added and centrifuged again at 10 000 x g for one minute. The flow-through was again discarded and the spin column was placed back into the collection tube. Nothing was added and the samples were centrifuged for a further minute at 10 000 x g. Finally, to elute the DNA, the spin column was placed into a clean 1.5 ml Eppendorf® tube and 50 µl ddH_2O was added. This was followed by centrifugation

at 10 000 x g for one minute. The purified PCR product was stored at 4°C until it was needed for sequencing.

Prior to sequencing the Del fragments it was required to purify the fragments in the gel as they had been sliced from a 2% agarose gel. The purification of the DNA from the fragments in the gel involved the use of a GFX PCR DNA and gel band purification kit [Amersham Biosciences, Buckinghamshire, England]. A gel slice was removed from the 2% agarose gel and cut into smaller pieces using a clean razor blade. The pieces were then placed into a 1.5 ml Eppendorf® tube to determine the weight of the gel slice to the nearest 10 mg (maximum limit of 300 mg). 10 µl of capture buffer for each 10 mg of gel slice was added to the 1.5 ml Eppendorf® tube. The tube was vortexed to mix the contents before placing it in a heating block at 60°C for approximately 15 minutes, to allow for the agarose to completely dissolve. To collect the sample at the bottom of the tube it was centrifuged briefly, after the agarose had melted. The sample was then transferred to a GFX column, which had been place in a collection tube, and left to incubate at room temperature for one minute. The flow-through was discarded from the collection tube after centrifugation at full speed in a microcentrifuge for 30 seconds. The GFX column was placed back into the collection tube and 500 µl of wash buffer was added. The sample was then centrifuged again at full speed for 30 seconds. The collection tube and flow-through were both removed, whereas the GFX column was placed in a clean 1.5 ml Eppendorf® tube. To elute the DNA, 30 µl of ddH_2O was added to the column. This was left to incubate at room temperature for one minute. Finally the sample was centrifuged at full speed for one minute to recover the purified PCR product.

Before the sequencing reaction could be performed the concentration of the purified PCR products had to be determined. This was accomplished by the electrophoresis of a horizontal 2% (w/v) agarose gel containing 0.01% (v/v) EtBr for staining purposes. A λ DNA concentration series, covering the range of 40 ng – 100 ng was prepared. Five microliters of the purified PCR product was mixed with 5 µl loading buffer and loaded onto the gel, which was resolved at 60 V for approximately one hour in 1X TBE buffer and visualized via UV light and photographed using the GelDoc system. The concentrations of the purified PCR products were estimated and where necessary the purified amplicons were diluted with ddH_2O to obtain the correct concentration (Table 3.3).

Table 3.3: Concentration of DNA required for sequencing according to PCR product size

PCR Product Size (bp)	Concentration Required (ng/µl)
100 – 200	1
200 – 500	3.3
500 – 1 000	6.6
1 000 – 2 000	13.3

Automated DNA sequencing was performed according to the chain termination protocol using a Perkin Elmer BigDye™ Terminator Cycle Sequencing kit [PE Applied Biosystems, Warrington WA, Great Britain]. The purified PCR products were used as templates in the sequencing reaction together with the same primers that were used in the PCR reaction earlier (Table 3.1 and Table 3.2). To 3 µl of the purified and diluted PCR product, 4 µl of the sequencing reaction solution was added to make a final reaction volume of 7 µl in 0.2 ml thin wall PCR tubes. This sequencing reaction solution contained 1 µl termination ready reaction mix, 2 µl ddH_2O and 1 µl of either the forward or reverse primer (1.1 pmol/µl). Cycle sequencing followed, conducted in a Perkin Elmer GeneAmp PCR system 9600 kit [PE Applied Biosystems, Warrington WA, Great Britain]. An initial denaturation at 96°C for two minutes was followed by 25 cycles consisting of denaturation for ten seconds at 96°C, annealing at 55°C for five seconds and an extension at 60°C for four minutes.

The products were loaded onto the ABI 3100 automated sequencer [PE Applied Biosystems, Warrington WA, Great Britain]. Analysis of electropherograms, following electrophoresis, was performed using the programs Sequence Analysis (V3.07) and BioEdit Sequence Alignment Editor (Hall, 1999). The sequences were compared with the sequence obtained for *GJB2* (accession number: NM_004004), *GJB6* (accession number: NM_006783) and clone RP11-501K3 on chromosome 13 (accession number: AL355984) from the NCBI Database.

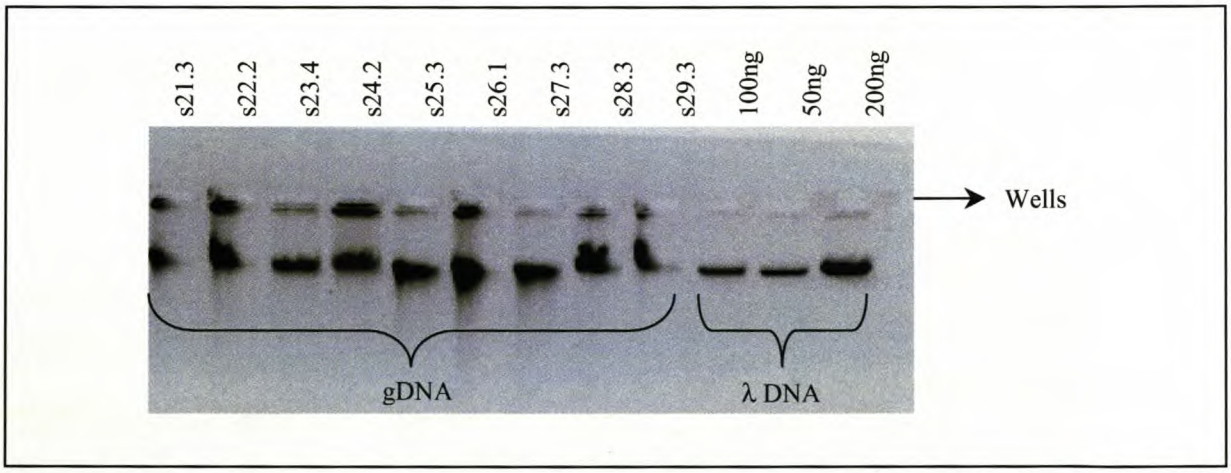
CHAPTER 4: RESULTS AND DISCUSSION

In this chapter the screening of *GJB2* and *GJB6* within South African families with non-syndromic autosomal hearing impairment as well as the comparison of different SSCP gel systems is presented. Results indicated in this chapter have been presented at several national and international conferences (Appendix B).

4.1 GENOMIC DNA QUANTIFICATION

To determine the concentration of the gDNA that was extracted from the whole blood, DNA samples were resolved by gel electrophoresis and the intensity of each band compared with a DNA concentration standard. This involved the loading of the extracted gDNA samples onto a 2% agarose gel together with a concentration gradient made up of λ DNA of varying concentrations, ranging from 50 – 200ng, for comparison purposes (Figure 4.1).

Figure 4.1: Representative photograph of an agarose gel used in gDNA quantification



A 2% agarose gel used for the determination of the concentration of extracted gDNA. The samples were resolved for one and a half hours at 100V in 1X TBE buffer and stained with EtBr. The first nine lanes contain samples of extracted gDNA whereas the remaining three lanes contain the λ DNA concentration gradient.

From the gel photographs it was determined that on average the aliquot that was loaded contained approximately 200 ng of extracted gDNA when compared with the λ DNA concentration gradient. It

was also determined from the 2% agarose gels that the samples contained high molecular weight DNA and there was no RNA present.

4.2 POLYMERASE CHAIN REACTION AMPLIFICATION

4.2.1 POLYMERASE CHAIN REACTION OF *GJB2* and *GJB6*

PCR amplification was conducted on gDNA to produce the overlapping fragments 1, 2, 4, 5 and 6 that cover the 678 bp of the coding exon 2 of *GJB2* (refer to Table 3.1 and Figure 3.1). Fragment 3 covered the entire coding region of *GJB2* and was used for automated DNA sequencing whereas the other fragments were used in SSCP analysis.

Fragments of Cx30 were produced by PCR amplification of gDNA. The fragment of 786 bp covered the entire coding exon of *GJB6* and was small enough to accomplish successful automated DNA sequencing in a single reaction. This gene was not screened using the different SSCP gel systems, therefore *GJB6* did not have to be amplified as a number of overlapping fragments. Amplification of *GJB6* was only conducted on those samples that had either one or both mutant alleles unidentified during *GJB2* analysis. PCR amplification was also used to amplify the Del fragment to screen for the Δ(*GJB6*-D13S1830) mutation, of *GJB6*. This fragment is only visible when the mutant allele is present in a patient as the large deletion allows for the primers to move within range of each other for amplification to occur. Refer to Table 3.2 and Figure 3.2 for the sequence and position information concerning the primers used to produce the Cx30 and Del fragments.

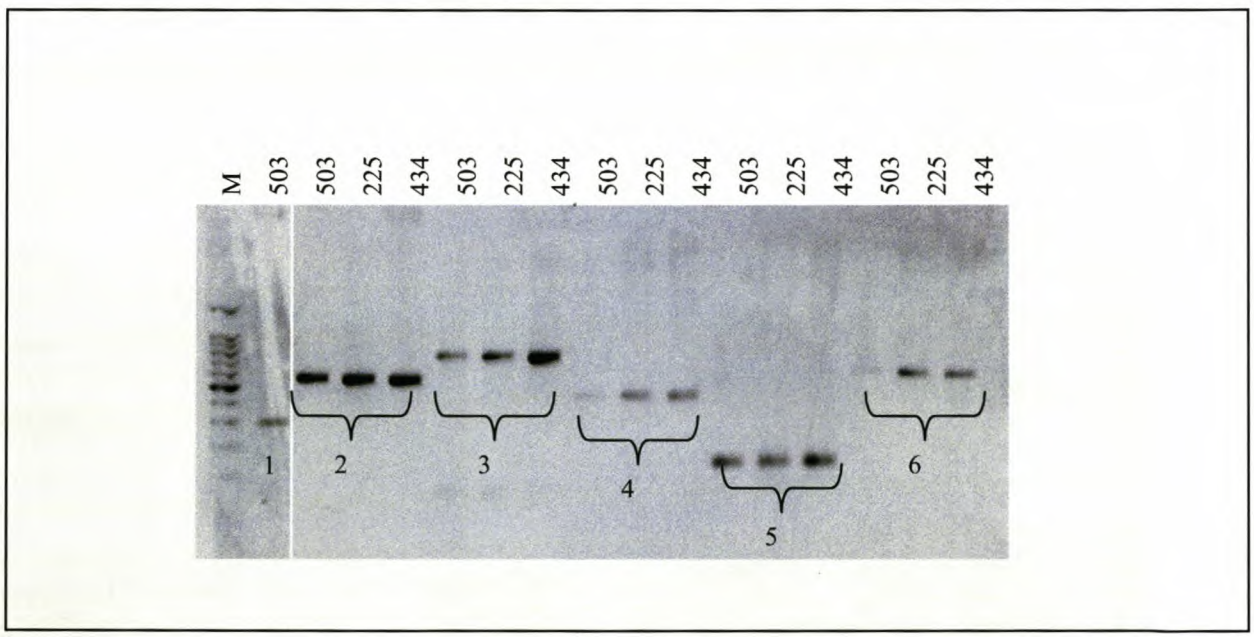
The annealing temperatures for the different primer sets were calculated using the equation reported by Thein and Wallace (1986). The optimized conditions, including the calculated and optimized annealing temperatures, as well as the fragment sizes produced by the primer sets are given it Table 4.1.

***Table 4.1: Optimized conditions for the fragments that covered* GJB2 *and* GJB6**

Gene Name	Fragment Name	Fragment Size (bp)	Calculated T_m of Forward Primer (°C)	Calculated T_m of Reverse Primer (°C)	Optimized T_m (°C)
GJB2	1	350	60	62	60
GJB2	2	600	64	62	60
GJB2	3	750	60	62	60
GJB2	4	490	64	60	66
GJB2	5	110	60	64	60
GJB2	6	600	64	62	66
GJB6	Cx30	850	58	54	56
GJB6	Del	700	68	72	50

All PCR amplicons for fragments 1 to 6 of *GJB2* were verified on a horizontal 2% agarose gel to determine if successful amplification had occurred (Figure 4.2) before proceeding with SSCP analysis or DNA sequencing. No negative controls are shown in Figure 4.2, as this gel was resolved only as an example of the various fragment sizes produced during PCR amplification of *GJB2*.

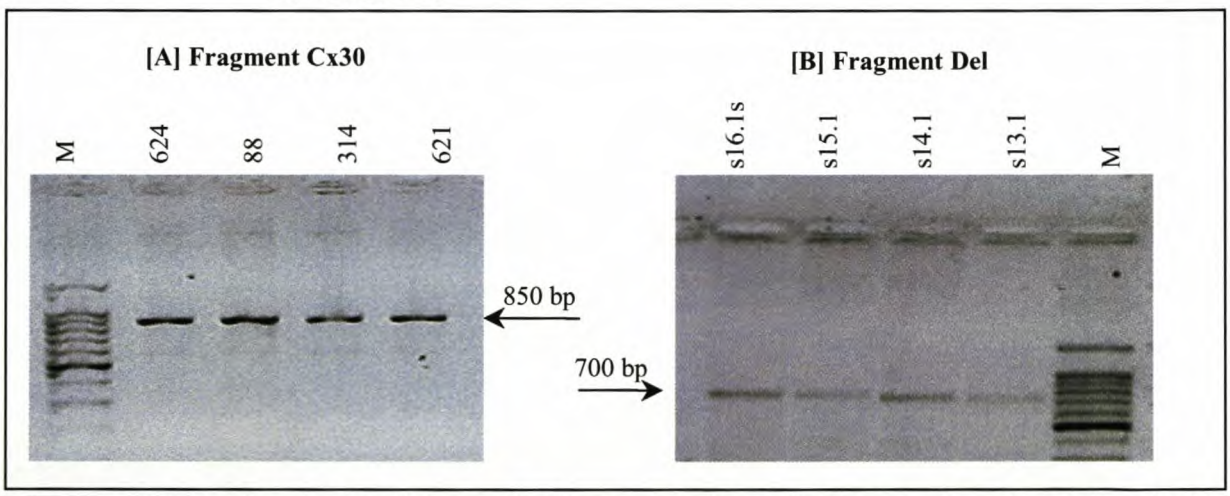
Figure 4.2: Representative photograph of PCR amplicons of* GJB2 *fragments 1 - 6



2% Agarose gel electrophoresis of fragments 1 – 6, which covers the coding region of *GJB2*, that were produced using PCR. The 2% agarose gel was resolved for 1 hour at 100V in 1X TBE buffer and stained with EtBr.
M = 100 bp DNA ladder.

Agarose gel electrophoresis was used to determine if PCR amplification had been successful in producing the amplicons of *GJB6*. In the case of the Del fragment, agarose gel electrophoresis was used for the detection of the absence or presence of the Δ(*GJB6*-D13S1830) mutation that involves the partial deletion of *GJB6* (Figures 4.3 A – B). Theoretically, this was possible due to the fragment only being produced in the presence of the large deletion as it resulted in the primers being within the necessary distance for amplification to occur. An amplicon should not be produced when the large deletion was not present, as the two primers would be located too far apart for successful amplification to occur. PCR amplification is not possible in the absence of the deletion because a limiting factor of this method is that it can only produce a fragment if the primers are within 0.1 – 5 kb of each other (Strachan and Read, 1999). Therefore, if the deletion is not present, the primers will be over 300 kb apart. No negative control was shown in Figure 4.3 A – B but they were resolved.

Figure 4.3 A – B: Representative photographs of PCR amplicons of* GJB6 *for fragments Cx30 and Del



Agarose gel electrophoresis of the PCR amplified fragments Cx30 and Del that cover the coding region of *GJB6* and the Δ(*GJB6*-D13S1830) mutation involving two different primer sets (Table 3.2 and Figure 3.2). The 2% agarose gels were electrophoresed for 1 hour at 100V in 1X TBE buffer and stained with EtBr. M = 100 bp DNA ladder.

4.3 COMPARISON OF SSCP GEL SYSTEMS

Three different SSCP gel electrophoresis systems were selected for testing for their effectiveness in detecting mutations in *GJB2*. The effectiveness of the system was determined by the ability of the

system to accurately detect the mutations present in *GJB2*. The three systems that were chosen included a mini PAGE system, a 12% (w/v) SSCP-urea system and a two buffer system. These methods were favoured as the equipment was readily available in the laboratory.

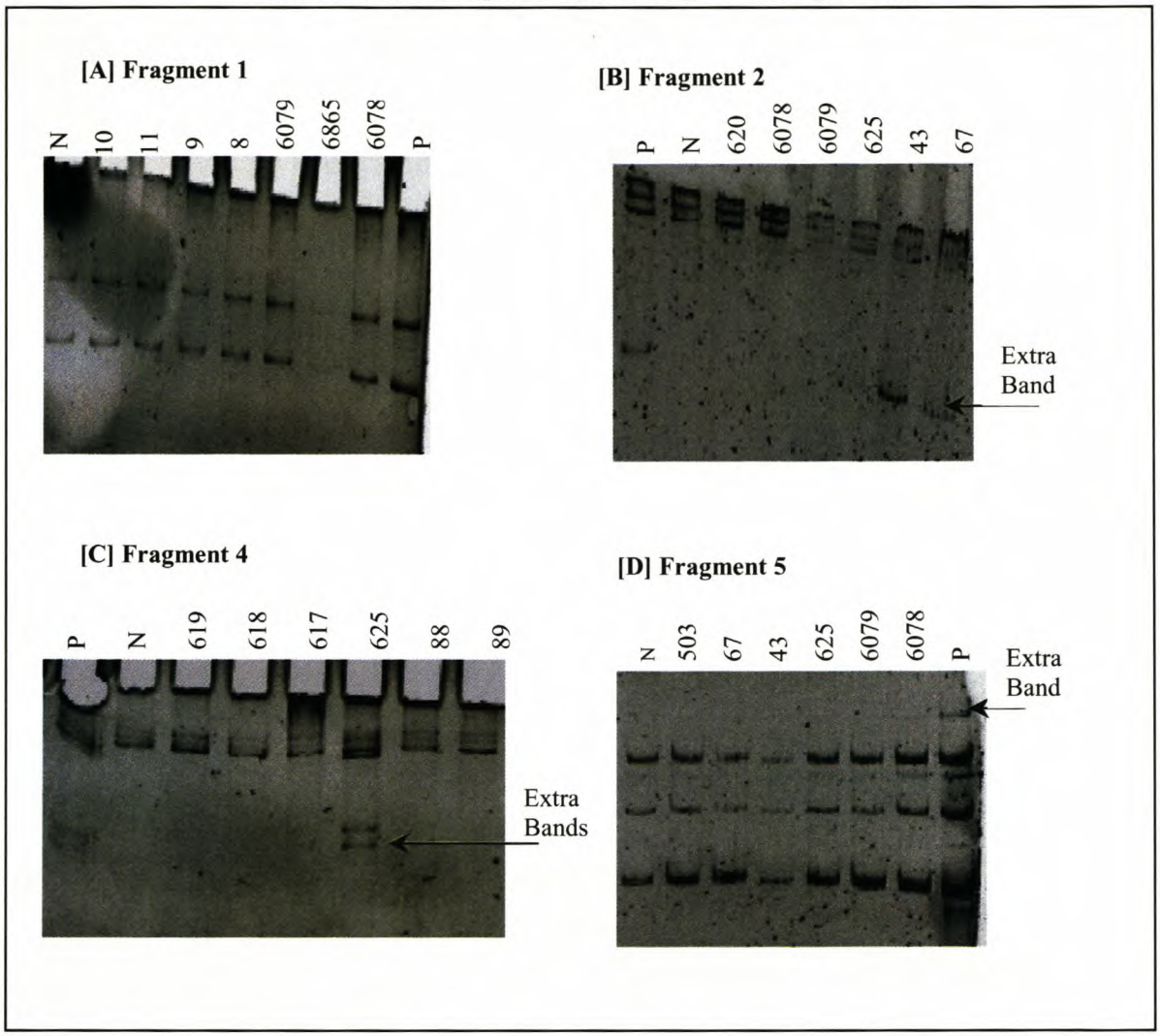
4.3.1 SINGLE-STRAND CONFORMATION POLYMORPHISM GEL ELECTROPHORESIS

4.3.1.1 MINI POLYACRYLAMIDE GEL ELECTROPHORESIS

The first SSCP system that was tested by identifying mutations in the coding exon 2 of *GJB2* was the mini PAGE technique. This method was conducted on the heat denatured PCR products of all the fragments except for fragment 6. It was assumed that if a mutation were present in this fragment it would not be visible because the digested pieces that make up fragment 6 are too similar in size to be separated on this gel system. Representative photographs of the gels obtained during mini PAGE analysis for each fragment, except fragment 6, are shown in Figure 4.4A - D. The positive controls that were used were samples that were known to contain specific mutations within the fragments whereas the negative controls were samples that were obtained from normal hearing individuals and were known to be negative for all disease causing mutations and benign polymorphisms within *GJB2*.

For fragment 1 (Figure 4.4A) no variation in banding pattern could be detected, even in the positive control, P, which was known to contain the 35delG mutation. This was unexpected, as there should have been a high frequency of variants present, including the common 35delG mutation located within this fragment. With the subsequent DNA sequencing of the samples it was determined that there were a large number of individuals who had been assigned negative status with mini PAGE analysis and who were in reality positive for the 35delG mutation. For example, in Figure 4.4A all the individuals appeared to be negative for any variants. With DNA sequencing it was determined that only sample 6079 was a wild type individual whereas samples 9, 6865 and 6078 were heterozygous carriers and samples 10 and 11 were homozygous for the 35delG mutation.

Figure 4.4A - D: Representative photographs of PCR amplification of* GJB2 *fragments analyzed by mini polyacrylamide gel electrophoresis



Mini polyacrylamide gel electrophoresis of fragments 1, 2, 4 and 5. The 10% polyacrylamide gels were resolved in 1X TBE buffer at 200V for four hours (fragments 1, 2 and 4) or two hours (fragment 5), and stained with EtBr. P = positive control and N = negative control.

It was, however, possible to detect variations in banding patterns in fragments 2, 4 and 5. Figure 4.4B contains an example of the results obtained for fragment 2. The positive control, P, was known to contain the 312del14 mutation, therefore producing the extra band lower on the gel during electrophoresis. It appeared that the mini PAGE analysis system was effective in detecting this specific mutation as samples 620, 625, 6078 and 6079 tested normal and samples 43 and 67 tested positive for 312del14. However, when these results were confirmed with automated DNA sequencing it was discovered that samples 625 and 6079, showing a low concentration on the gel, were actually

heterozygotic individuals, therefore indicating that equal quantities of PCR amplicons should be used. It was also observed that from these gels it was not possible to assign homozygous or heterozygous status for a specific mutation.

The positive control, P, in fragment 4 (Figure 4.4C) was also known to contain the 312del14 mutation as fragments 2 and 4 overlapped in this area of *GJB2*. The positive control produced two extra bands lower on the gel during electrophoresis using the mini PAGE system. Again it was thought that the mini PAGE system was effective in detecting the 312del14 mutation as it appeared that samples 619, 618, 617, 88 and 89 were wild type individuals and 625 was positive for the 312del14 variant. However, when these results were tested with automated DNA sequencing it was determined that samples 618 and 617 were not wild types but heterozygous individuals for mutation 312del14. Once again the low concentration of these two samples on the gel probably caused this phenomenon.

In fragment 5 (Figure 4.4D) the positive control, P, contains the V153I polymorphism. This variation was characterized by the extra band that was produced above the normal banding pattern on the gel during electrophoresis. It was assumed that this system is successful at detecting this variant as the positive control was clearly visible and those samples that appeared normal during gel electrophoresis were later confirmed as such during automated DNA sequencing. However, this needs to be tested further as none of the samples that were tested contained the V153I polymorphism and therefore further samples that are known to contain the variation need to be screened using this system.

4.3.1.2 SSCP-UREA GEL ELECTROPHORESIS

The second SSCP system that was tested was a 12% (w/v) PAA gel containing 7.5% (w/v) urea. This method was conducted on the heat denatured PCR products of all the fragments that cover exon 2 of *GJB2*. These samples were run on a larger gel (37 X 16.5 cm) so that electrophoresis could be performed for longer thereby allowing for the screening of fragment 6, which consisted of two relatively similar sized fragments. This system also allowed for the analysis of the dsDNA bands, as they were still present on the gel after electrophoresis. Representative photographs of the gels that were obtained of all the fragments during the SSCP-Urea gel analysis are presented in Figure 4.5A - E. The positive controls that were used were samples that were known to contain specific mutations within the fragments whereas the negative controls were samples that were obtained from wild type individuals

and were known to be absent for all disease causing mutations and benign polymorphisms within *GJB2*.

For fragment 1 (Figure 4.5A) it was not possible to detect any variation in band pattern. This was surprising since the detection of the common 35delG mutation was expected in the positive control, P. For example, it was later confirmed, with automated DNA sequencing, that the samples 230, 231 and 224 (Figure 4.5A) were actually heterozygous for the 35delG mutations and not wild type individuals as the SSCP-urea analysis had indicated.

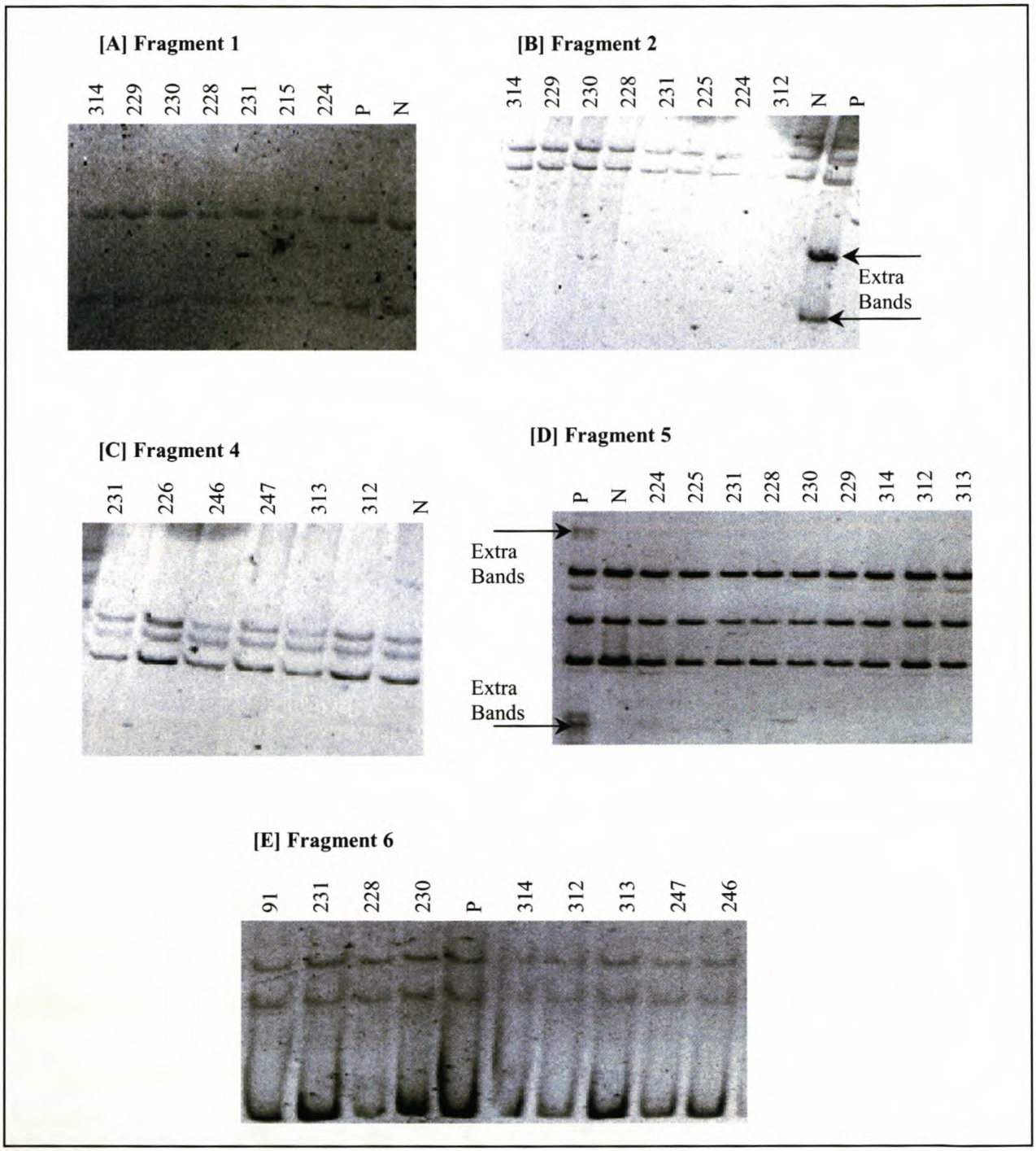
It was possible to identify the variation in banding pattern caused by mutations present in fragments 2, 4 and 5. In fragment 2 (Figure 4.5B) the mutation caused two extra bands to form lower down from the normal three bands as was shown by the positive control (P), which was known to contain the 312del14 mutation. All the samples that are indicated in this example were assigned as wild types during SSCP-urea analysis and this deletion variant was later confirmed with automated DNA sequencing to be accurate.

The example of fragment 4 shown in Figure 4.5C indicates all the samples to be wild types for the expected mutation, 312del14. Subsequent confirmation using automated DNA sequencing determined these results to be correct. The SSCP-urea system was able to detect the 312del14 mutation even though this is not indicated in this example.

In fragment 5 (Figure 4.5D), the positive control (P) was known to contain the V153I polymorphism. The variation was visible during gel electrophoresis as extra bands above and below the normal band pattern. It appears that this system was successful in detecting this variation, as the positive control was clearly distinguishable from the negative control (N).

The positive control, P, in fragment 6 (Figure 4.5E) was also known to contain the 312del14 mutation as this fragment overlapped with fragment 2 and 4. However, using this system it was not possible to detect a variation between the positive control and those samples that were wild type.

Figure 4.5A - E: Representative photographs of SSCP-urea gel electrophoresis of* GJB2 *fragments



SSCP-urea gel electrophoresis of fragments 1, 2, 4, 5 and 6. The 12% polyacrylamide gels were resolved in 0.5X and 1.5X TBE buffer at 300V for 18 hours (fragments 1, 2, 4 and 6) or seven hours (fragment 5). They were stained with EtBr. P = positive control and N = negative control.

4.3.1.3 TWO BUFFER POLYACRYLAMIDE GEL ELECTROPHORESIS

The final SSCP analysis technique tested was the two buffer gel electrophoresis system. Again a 12% (w/v) PAA gel was used but this time it contained 7.5% (w/v) glycerol instead of 7.5% (w/v) urea. Another difference was that it was run in Tris-Formate and Tris-Borate buffer instead of 0.5X TBE and 1.5X TBE. This method was used for the analysis of heat denatured PCR products of all the fragments of *GJB2* and the gel apparatus used produced gels that were 30 cm long. Figures 4.6A - D are representative photographs of the gels obtained for all the fragments that were screened using this system. The positive controls that were used were samples that were known to contain specific mutations within the fragments whereas the negative controls were samples that were obtained from normal hearing individuals and were known to be absent for all disease causing mutations and benign polymorphisms within *GJB2*.

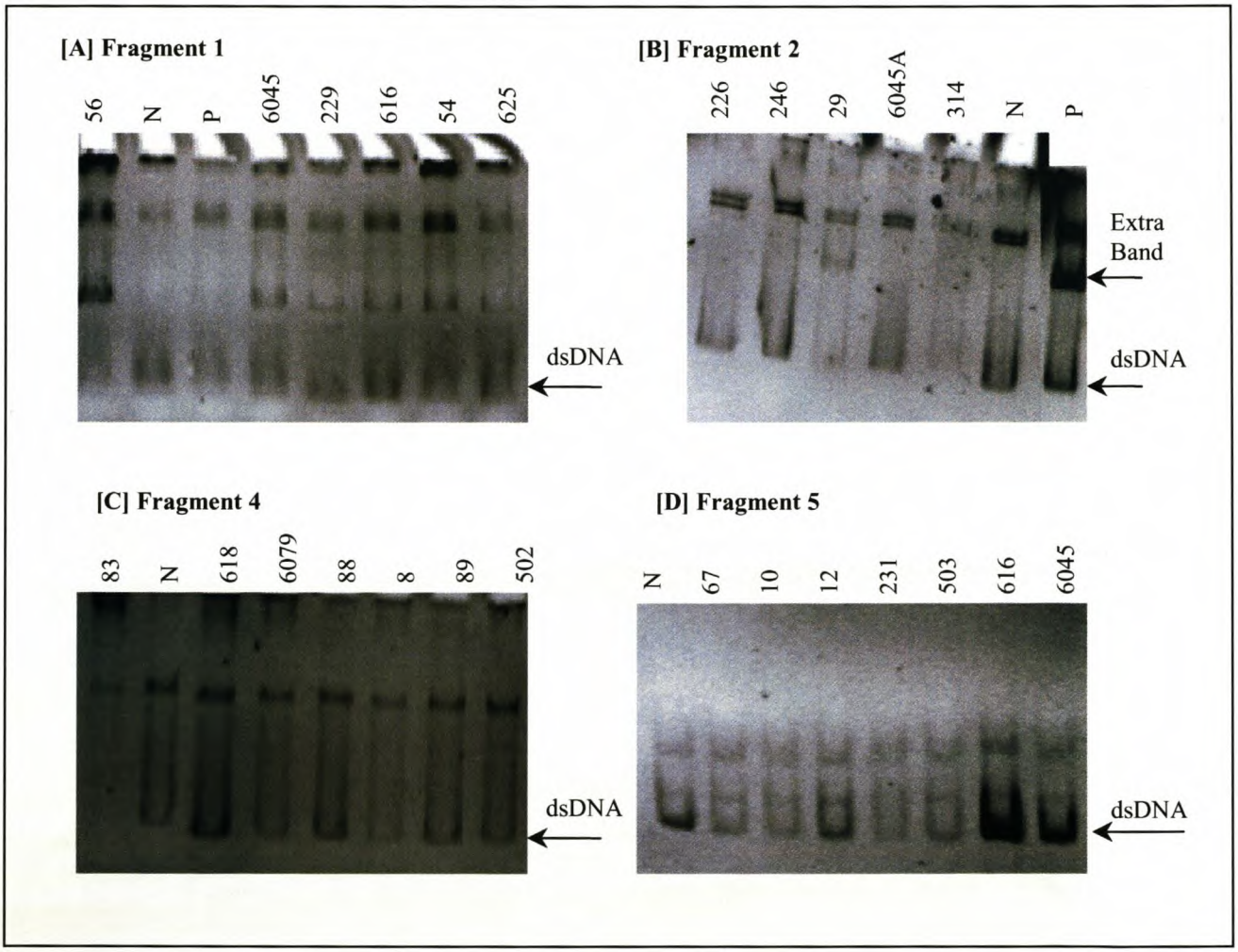
Using the two buffer SSCP analysis system, it was once again not possible to identify any variations in band pattern resulting from mutations present in fragment 1 (Figure 4.6A). Even the positive control (P) that was known to contain the 35delG mutation appeared to be wild type. With automated DNA sequencing it was determined that, in this example, samples 56, 616 and 625 were in fact heterozygous individuals for the 35delG mutation.

For fragment 2 a positive control (P) containing the 312del14 mutation was used. In Figure 4.6B it was observed that the two buffer SSCP system was able to detect this mutation as the variation of an extra band was visible in both the positive control and sample 29. Automated DNA sequencing later determined that this result was correct however it also determined that sample 31 was a homozygous individual for the 312del14 mutation, which was not detected using the two buffer SSCP system.

In fragment 4 a positive control (P) for the 312del14 mutation was used and the variation was visible although it is not shown in this example (Figure 4.6C). Even though the positive control was detected successfully, the variation was not always clear as was observed with sample 618, which appeared normal during two buffer SSCP analysis, but was determined to be a heterozygous individual with automated DNA sequencing.

The gels containing fragment 5 (Figure 4.6D) showed no variation in banding pattern. Even the positive control, P, (not shown in this example) that was known to contain the V153I polymorphism showed no distinct difference from those samples that contain the wild type allele. This was an unexpected result as this polymorphism was detected with all the other gel systems used.

Figure 4.6A - D: Representative photographs of two buffer gel electrophoresis analysis of GJB2 fragments



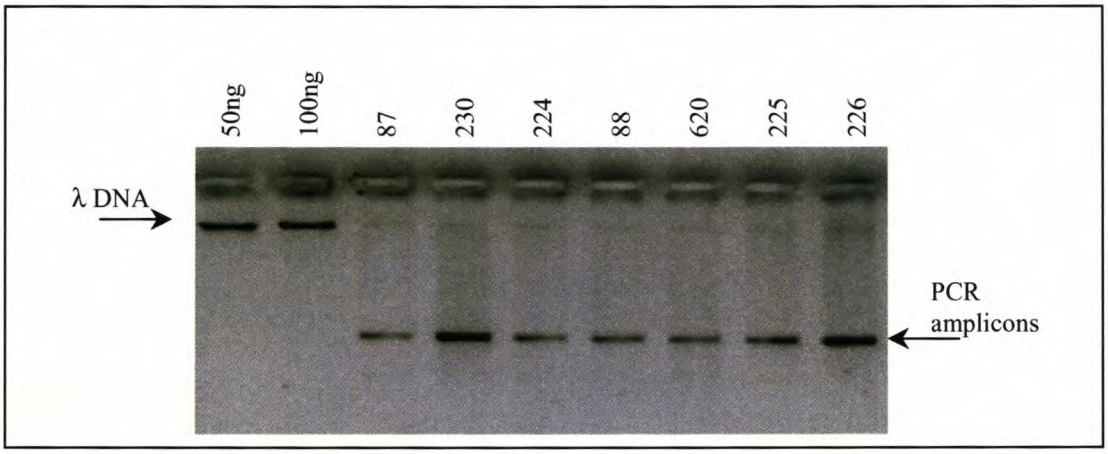
Two buffer SSCP gel electrophoresis of fragments 1, 2, 4 and 5. The 12% polyacrylamide gels were resolved in Tris-Formate and Tris-Borate buffer at 350V for two hours and were stained with EtBr. P = positive control and N = negative control.

4.4 AUTOMATED DNA SEQUENCING

4.4.1 DNA CONCENTRATION DETERMINATION OF AMPLICONS

The concentration of the PCR amplicons corresponding to both *GJB2* and *GJB6* had to be determined before automated DNA sequencing could commence. This was performed because a specific concentration of the PCR amplicon was required (dependent on the size of the fragment) for successful sequencing (Table 3.3). To achieve this a 2% agarose gel was used, whereby a concentration gradient of λ DNA, ranging from 50 – 100 ng, was resolved together with the PCR amplicons (Figure 4.7).

Figure 4.7: Representative photograph of the quantification of amplicons



A 2% agarose gel used for the quantification of the PCR amplicons. The gel was resolved for two hour at 120V in 1X TBE buffer and stained with EtBr. The first four lanes contain the λ DNA concentration gradient whereas, the remaining lanes contain samples of PCR amplicons to be sequenced.

In the case of fragments 1 and 5 the final concentration of the PCR amplicons had to be 3.3 ng/ul before successful DNA sequencing could take place whereas, for fragments 2, 3, 4, 6, Cx30 and Del the concentration needed was 6.6 ng/ul. Therefore, those samples that contained a higher PCR amplicon concentration were first diluted to the required concentration before DNA sequencing.

4.4.2 AUTOMATED DNA SEQUENCING OF SSCP GEL ELECTROPHORESIS VARIANTS

Automated DNA sequencing was first conducted on all the PCR products of fragment 1 of *GJB2* due to no results being obtained during SSCP analysis. It was expected that there would be a high frequency of individuals with the most common mutation, 35delG, which causes non-syndromic autosomal recessive hearing loss. The specific results pertaining to this mutation are discussed later in section 4.4.3.1.

DNA sequencing was also conducted on the samples that showed a variation in SSCP band patterns, due to the presence of a possible mutation. The mutation was confirmed by DNA sequencing to be the 312del14 mutation that is found in both fragment 2 and 4 due to these fragments overlapping in this area of the sequence. Again the specific results regarding this mutation are discussed later in section 4.4.3.2.

4.4.3 SCREENING OF *GJB2*

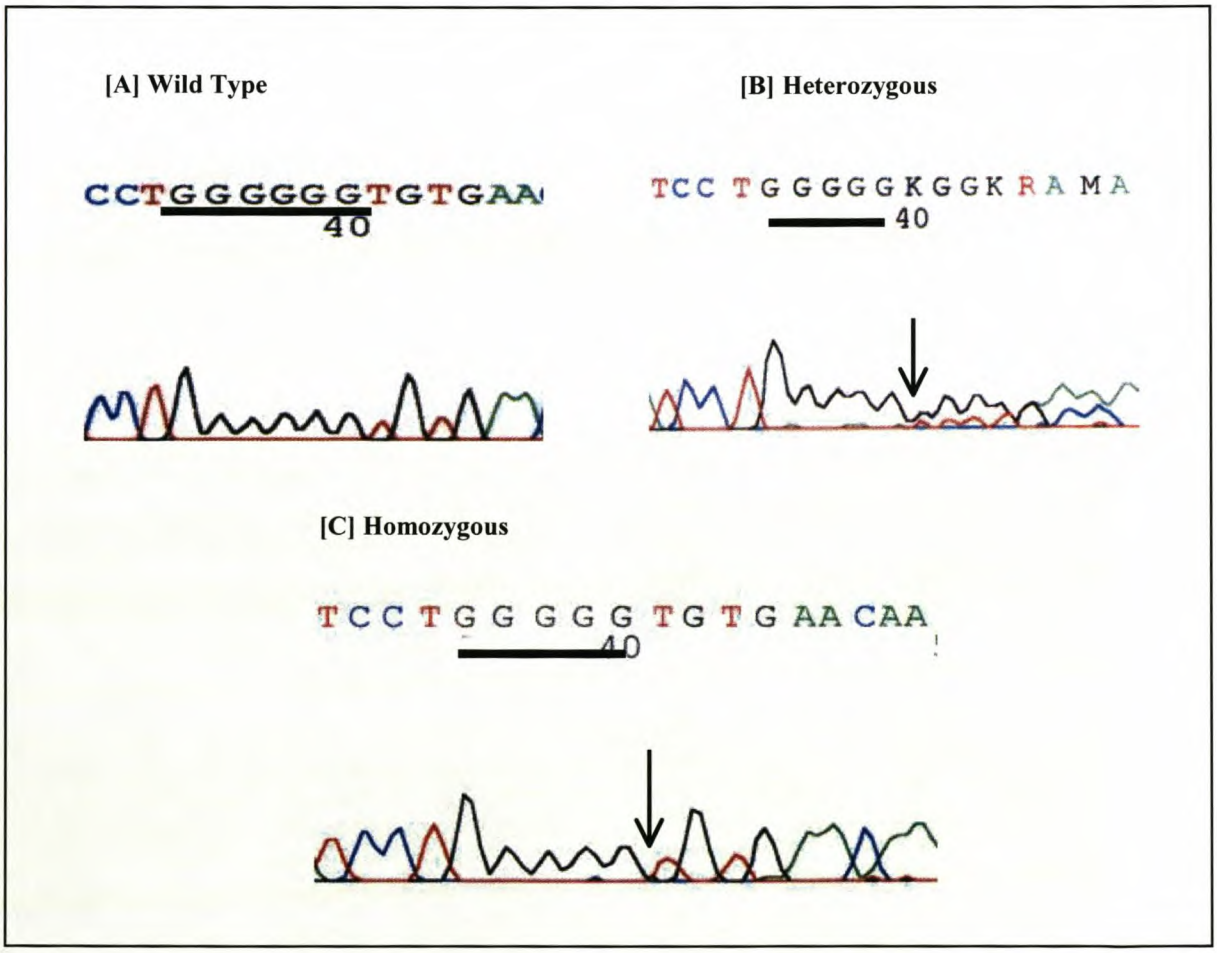
It was decided that any further analysis of samples obtained from families with non-syndromic autosomal deafness would be conducted using only automated DNA sequencing of *GJB2* for possible mutations. The reasons for this decision were firstly, that it was noted that none of the SSCP gel electrophoresis systems were successful in detecting all the mutations within the *GJB2* gene. Secondly, the only way to detect the common 35delG mutation was by automated DNA sequencing. Finally, those samples that showed a change in mobility during SSCP analysis had to be sequenced to determine the specific mutation that was present.

A total of seven different disease-causing mutations and four benign polymorphisms were detected in the *GJB2* gene. These mutations included the common Caucasian mutation, 35delG as well as a novel mutation, N62I. All the mutations and polymorphisms that were encountered are discussed in further detail in the following sections.

4.4.3.1 THE 35delG MUTATION

The 35delG mutation of *GJB2* was detected in homozygous form, 35delG/ 35delG, (Figure 4.8C) in three familial and one sporadic family. It was also encountered in a compound heterozygous form (Figure 4.8B) in three familial and four sporadic families. In compound heterozygote state it was observed together with other *GJB2* mutations, which included 312del14, W24X and N62I. None of the control samples from either the Caucasian or Mixed Ancestry populations had any individuals harbouring the 35delG mutation. Refer to Section 4.5.1 for further information concerning the statistical analysis of the 35delG mutation with regards to the frequencies of this mutation within the different study groups.

Figure 4.8 A - C: Representative electropherograms of the* GJB2 *mutation 35delG



The 35delG variant is a recessive mutation that is caused by a deletion of a single G from a set of six found at nucleotide position 30 to 35. This deletion results in a frameshift mutation that leads to a Valine substituting for a Glycine at amino acid 12, followed by a premature stop codon at nucleotide 38 (amino acid 13). This causes the formation of a significantly shorter protein that is unlikely to be inserted into the plasma membrane as this mutation lies within the N-terminus region of the connexin 26 protein.

For more information concerning the 35delG mutation, including the frequency of this mutation within other populations as well as the origin of the mutation, refer to Section 2.5.4.1.

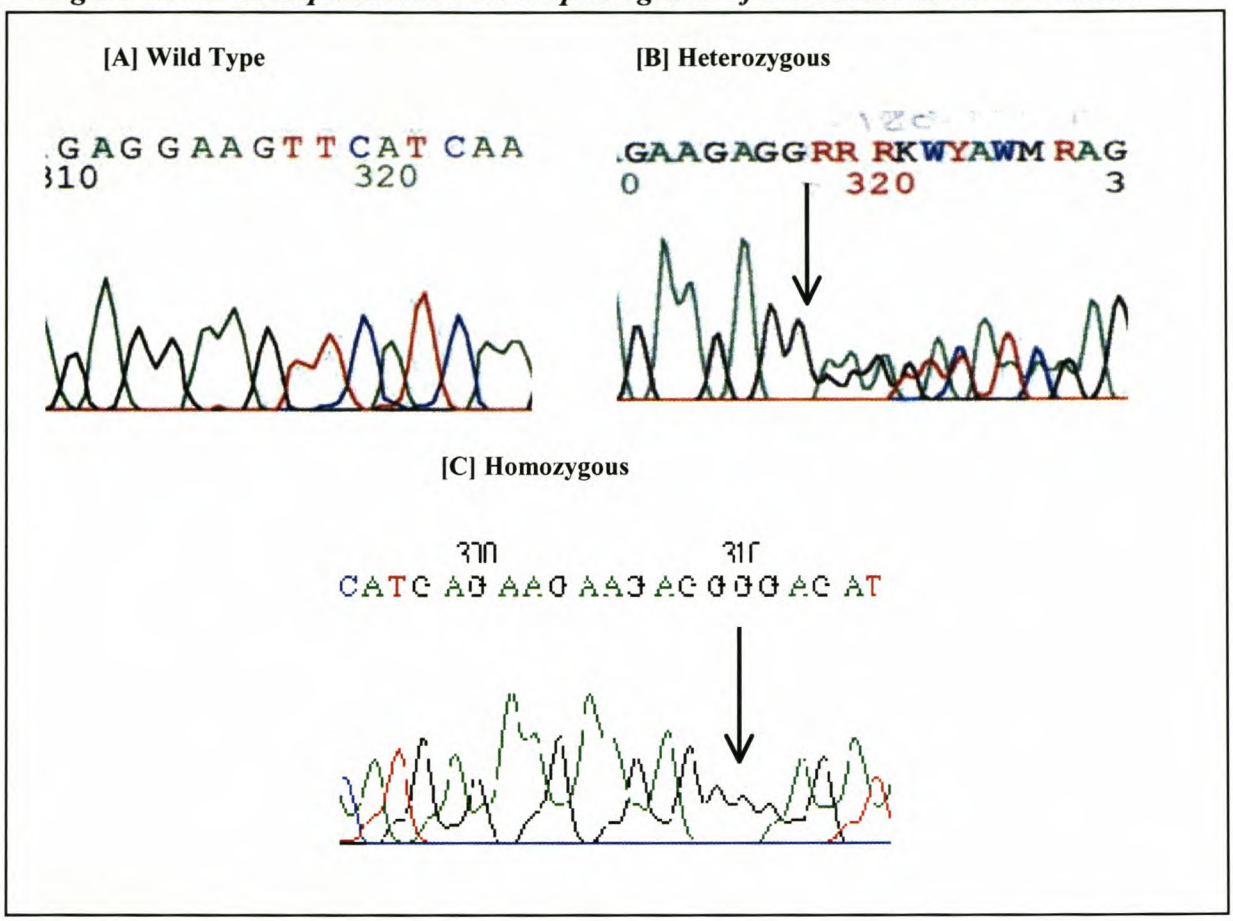
4.4.3.2 THE 312del14 MUTATION

The 312del14 variant is a recessive mutation that was detected in *GJB2* as the result of the deletion of 14 nucleotides starting at position 312, leading to a frameshift mutation that eventually forms a premature stop codon. This mutation results in a significantly shorter Cx26 protein that is unable to perform its function as the mutation lies within the cytoplasmic loop.

The 312del14 mutation of *GJB2* was identified in homozygous form, 312del14/ 312del14, (Figure 4.9B) in two familial and one sporadic family. It was also encountered in compound heterozygous form (Figure 4.9C) in two familial and four sporadic families. In compound heterozygote state it was only encountered with the *GJB2* mutation 35delG. This mutation was only detected in heterozygous form in a single Caucasian individual from the control panel.

The 312del14 mutation was first reported in 2000 by Rabionet *et al.*, in a Spanish and an Italian population. However, he assumed that this mutation was possibly the same as a previously reported mutation, 310del14, which was found in the same protein region. In 2001, Lin *et al.* also detected the 312del14 mutation in a study involving an American population from various backgrounds. Here the 312del14 mutation was found in a heterozygous state at a frequency of 0.026 amongst those with non-syndromic recessive hearing loss and therefore was considered as a separate recessive mutation from the previously described 310del14. Refer to Section 4.5.1 for further information concerning the statistical analysis of the 312del14 mutation with regards to the frequencies that this mutation was detected in the different study groups.

Figure 4.9 A - C: Representative electropherograms of the* GJB2 *mutation 312del14

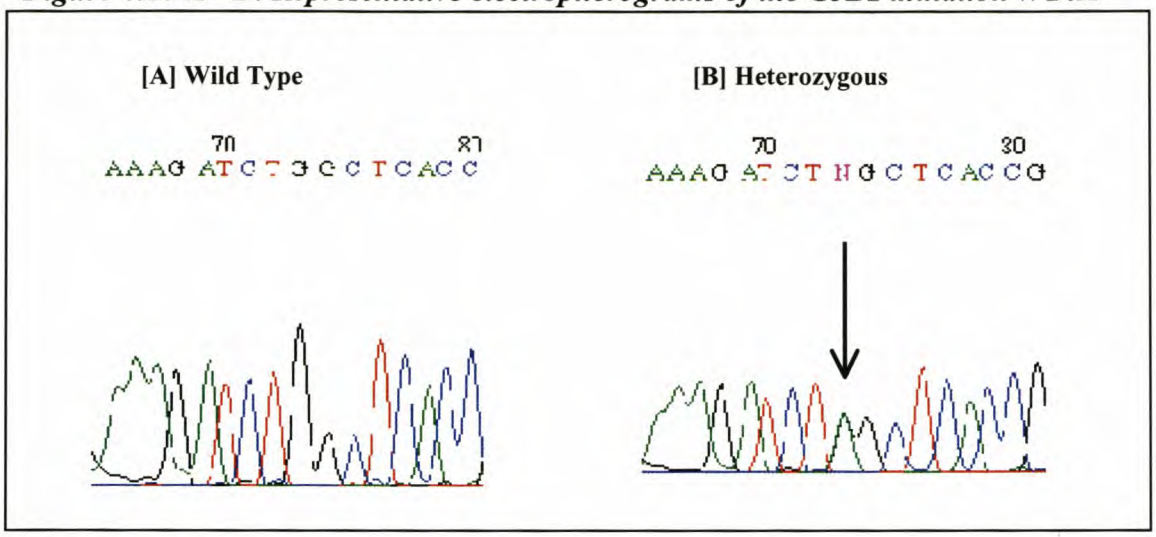


4.4.3.3 THE W24X MUTATION

The W24X mutation of *GJB2* was only detected twice in this study. It was identified in a compound heterozygous form (Figure 4.10B) together with the *GJB2* mutation 35delG in a sporadic family of Mixed Ancestry. The second case was in an individual from the Mixed Ancestry control group (Section 4.5.1).

The W24X mutation that was detected in *GJB2* was a recessive nonsense mutation, which was the result of the conversion of a G to an A at nucleotide position 71. This mutation was caused by the conversion of a non-polar, hydrophobic Tryptophan (TGG) into a stop codon (TGA) at the amino acid position 24. The W24X mutation produces a significantly shorter Cx26 protein that is unlikely to be inserted into the plasma membrane since the mutation lies within the first transmembrane domain.

Figure 4.10 A - B: Representative electropherograms of the GJB2 mutation W24X



The W24X mutation was first detected in two Pakistani families in 1997 (Kelsell *et al.*, 1997). This was followed shortly by its detection in two Indian families in a homozygous as well as a compound heterozygous state. It was noticed that the W24X allele was always associated with the same closely linked marker alleles and therefore the mutation may have arisen from a single ancestral variation (Scott *et al.*, 1998B). The W24X mutation has been reported in a heterozygous state at a frequency of 0.026 amongst an American population, of varied ancestry, suffering from non-syndromic autosomal recessive deafness (Lin *et al.*, 2001). The W24X variant has also been reported in the Spanish and Italian populations (Rabionet *et al.*, 2000). However, it appears to be predominantly found in individuals from the Indian subcontinent (India, Pakistan and Bangladesh). This, therefore, suggests that the W24X mutation may be a common mutation to this ethnic group (Rickard *et al.*, 2001). Refer to Section 4.5.1 for further information concerning the statistical analysis of the W24X mutation with regards to the frequencies that this mutation was detected within the different study groups.

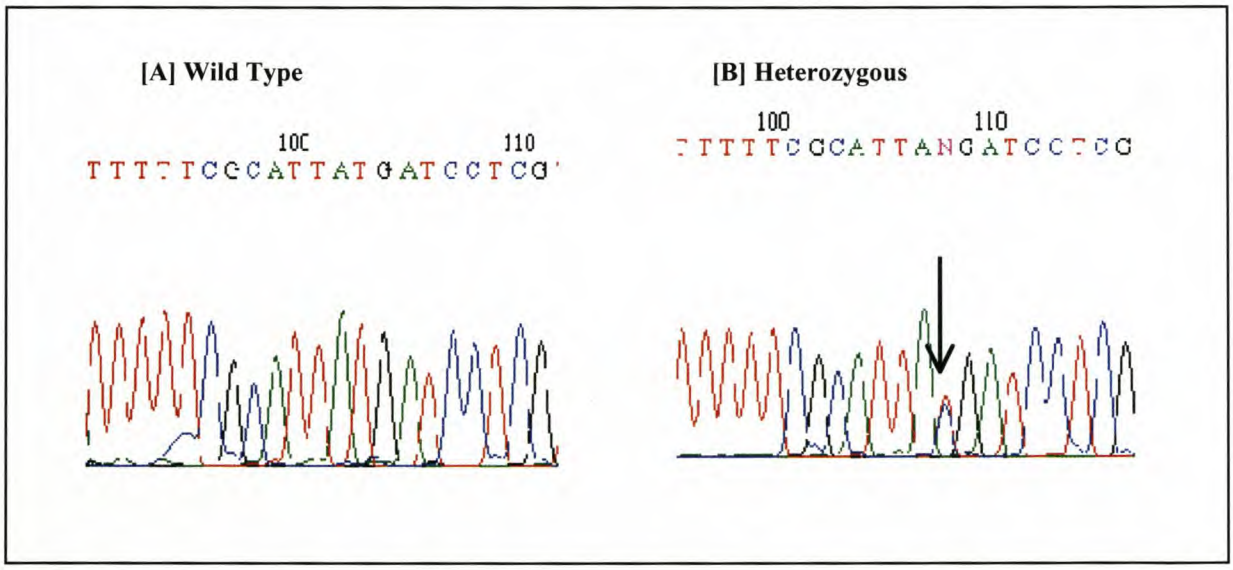
4.4.3.4 THE M34T MUTATION

The M34T mutation of *GJB2* was only detected in a single individual from the Caucasian control group and therefore it was only encountered in its heterozygous form (Figure 4.11B).

The M34T mutation that was detected in *GJB2* was a recessive missense variant that was the result of the conversion of a T to a C at nucleotide position 101. This mutation caused the conversion of a non-polar, hydrophobic Methionine (ATG) into a polar, hydrophilic Threonine (ACG) at amino acid

position 34 but still resulted in a full-length product. The M34T mutation was assumed to alter the shape of the first transmembrane domain due to a large amino acid being replaced by a smaller one (Kelsell *et al.*, 1997). It was determined that this altered protein conformation, caused by the M35T variant, hindered radial oligomerization of the subunits into connexons. A resultant decrease in the number of homomeric M34T connexons was noted due to connexon movement from the intracellular space to the plasma membrane requiring oligomerization. Those connexons that did form were functionally abnormal as their voltage sensor gating was changed (Prasad *et al.*, 2000). It was determined that the Methionine amino acid at codon 34 played an important role in connexin functioning as this residue was conserved in Cx26 and Cx32 of different species (Kelsell *et al.*, 1997).

Figure 4.11 A - B: Representative electropherograms of the* GJB2 *mutation M34T



Kelsell *et al.* first detected the M34T variant in 1997, and described it as causing an autosomal dominant form of hearing loss. Since then there has been continued debate as to whether this variant was the cause of autosomal dominant or recessive deafness or even possibly a benign polymorphism. A number of studies, which are discussed briefly below, have supported the various theories. However, according to the Connexin Homepage (2003) the M34T variant is classified as a mutation that is responsible for non-syndromic recessive deafness.

After the discovery of the M34T variant in 1997, Kelley *et al.* (1998) also detected it amongst an American population group and described it as a recessive mutation since it was observed amongst individuals with normal hearing. However, the possibility that it was a polymorphism was not ruled out

due to the fact that no homozygous individuals had yet been identified. Three possible explanations were given as to how the M34T variant can be present in an individual with no signs of hearing impairment. They were as follows (Scott *et al.*, 1998B):

- The M34T variant was possibly the cause of non-syndromic recessive deafness in only some individuals and not expressed in all, however this was unlikely due to a number of individuals with normal hearing being hetetrozygous carriers.
- Some individuals were possibly carrying a change that compensated for the effect of the M34T variant, however no such change had been identified.
- The M34T variant was a polymorphism that was only found within a small percentage of the general population.

A study involving the injection of *Xenopus laevis* oocytes with *in vitro*-transcribed complementary RNA that encoded either wild type human Cx26, M34T Cx26 or a combination of both was used to determine the role of the M34T variant in non-syndromic deafness. It was shown that the M34T variant did not bring about coupling between paired oocytes and the combination of both the M34T variant and the wild type produced an inhibition in intercellular coupling. Therefore, the M34T variant caused hearing loss by dominant inhibition of the activity of the wild type Cx26 (White *et al.*, 1998).

Finally, in 2001 a homozygous individual for the M34T variant was identified amongst a sample population collected from the UK and Ireland who suffered from non-syndromic autosomal recessive hearing loss. Due to the identification of a M34T/M34T individual with mild hearing loss it was determined that the M34T variant is responsible for non-syndromic autosomal recessive mild hearing loss (Houseman *et al.*, 2001). Even with this discovery that seemed to finally classify the M34T variant, in the same year, two individuals in a French population with normal hearing were discovered to harbour the M34T/35delG genotype. This again, indicated that the M34T allele was a benign polymorphism (Marlin *et al.*, 2001). Currently, the M34T variant is classified as a recessive mutation that leads to non-syndromic hearing impairment (Connexin Homepage, 2003).

Table 4.2: Frequency of the M34T allele of* GJB2 *in various populations

Population	M34T Frequency (Deaf Individuals)	M34T Frequency (Control Individuals)	Reference
Spanish/Italian	0.001	------	Houseman *et al.*, 2001
Japanese	0.0	0.0	Houseman *et al.*, 2001
French	0.0	------	Houseman *et al.*, 2001
French	------	0.009	Marlin *et al.*, 2001
Israeli	0.0	------	Houseman *et al.*, 2001
British/Irish	0.032	0.020	Houseman *et al.*, 2001
Australian	0.100	------	Wilcox *et al.*, 2000
American	------	0.015	Kelley *et al.*, 1998
American	------	0.025	Green *et al.*, 1999
American	0.042	0.023	Prasad *et al.*, 2000
American	0.080	------	Kenna *et al.*, 2001
American	0.079	------	Lin *et al.*, 2001

The sample group sizes in some of the studies were small therefore, the estimated frequencies of the M34T allele should be treated with caution.

The frequency of the M34T variant has been determined amongst a number of different populations (Table 4.2). It appears to be most common amongst the American, British, Irish and Australian populations. However, in other populations such as the French, Spanish, Italian and Japanese it is present at a low frequency or not at all. This suggests that a single ancestral mutational event took place to form the M34T allele in either the UK or Ireland that was then carried over to the USA and Australia by colonization of these countries by the British (Houseman *et al.*, 2001). Refer to Section 4.5.1 for further information concerning the statistical analysis of the M34T mutation and the frequencies determined for this variation within this study.

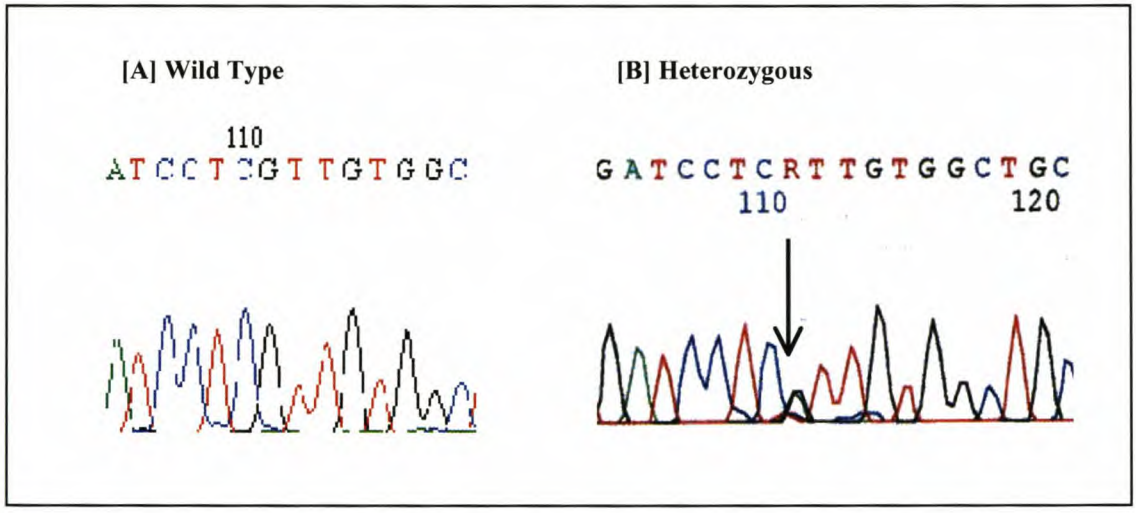
4.4.3.5 THE V37I MUTATION

The V37I mutation of *GJB2* was only detected in a single Indian sporadic family in a compound heterozygous patient (Figure 4.12B).

The V37I missense mutation that was detected in *GJB2* was the result of the conversion of a G to an A at nucleotide position 109. This mutation leads to the change from a non-polar, hydrophobic Valine (GTT) to a non-polar, hydrophobic Isoleucine (ATT) at the amino acid position 37. This Valine is

conserved amongst all β-connexins whereas, it is found as a Serine, Alanine or Glycine amongst the α-connexin family (Kelley *et al.*, 1998; Rabionet *et al.*, 2000). This indicates that this Valine is important for the correct functioning of Cx26, which is a β-connexin. The V37I mutation most likely alters the shape of the first transmembrane domain due to the difference in size of the amino acids.

Figure 4.12 A - B: Representative electropherograms of the* GJB2 *mutation V37I



At first there was uncertainty as to the effect of the V37I variant. When it was first detected it was classified as a benign polymorphism due to it only being detected once amongst a control group of individuals with normal hearing (Kelley *et al.*, 1999). In 2000, Abe *et al.*, reported that the V37I variant frequently cosegregated, in a Japanese population, with non-syndromic recessive hearing loss and therefore it was possible that this variant could be disease-causing. At the same time a homozygous individual with non-syndromic autosomal recessive deafness was reported in the USA. However, there was still confusion because in the same study, a family was identified where both parents with normal hearing had genotypes V37I/V27I and V37I/Wild Type. Their two deaf children had the genotypes V37I/V37I and V27I/Wild Type respectively. This therefore, suggested that neither mutation caused deafness and that one child (V27I/Wild Type) represented a phenocopy (Prasad *et al.*, 2000). Further examples of homozygous individuals for the V37I variant were identified in Spanish/Italian and Australian populations and the V37I allele was thus described as a recessive mutation that causes mild non-syndromic autosomal recessive deafness (Rabionet *et al.*, 2000 and Wilcox *et al.*, 2000). The Connexin Homepage (2003) currently confirms this classification of the V37I variant.

The frequency of the V37I variant has been determined in a few populations, including the Japanese, American, Australian and French (Table 4.3). The V37I variant is found at relatively high frequencies amongst all these groups. These high frequencies can be due to some of the studies using relatively small sample groups. Refer to Section 4.5.1 for further information concerning the statistical analysis of the V37I mutation with regards to the frequencies at which this variation was detected within this study.

Table 4.3: Frequency of the V37I allele of* GJB2 *in various populations

Population	V37I Frequency (Deaf Individuals)	V37I Frequency (Control Individuals)	Reference
Japanese	0.057	0.010	Abe *et al.*, 2000
Japanese	------	0.030	Kudo *et al.*, 2000
Japanese	0.014	0.010 – 0.030	Marlin *et al.*, 2001
American	0.017	------	Prasad *et al.*, 2000
American	Homozygous = 0.079 Heterozygous = 0.052	------	Lin *et al.*, 2001
Australian	0.150	------	Wilcox *et al.*, 2000
French	0.036	0.009	Marlin *et al.*, 2001

4.4.3.6 THE W44X MUTATION

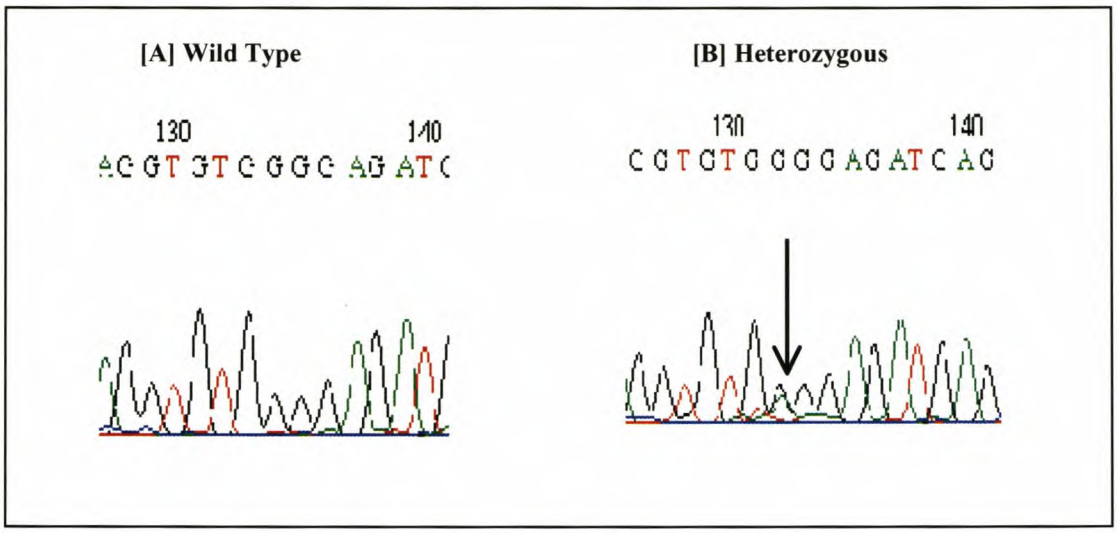
The W44X mutation of *GJB2* was only detected in a single individual within the Caucasian control group (Section 4.5.1) in its heterozygous form (Figure 4.13B).

The W44X nonsense mutation that was detected in *GJB2* was the result of the conversion of a G to an A at nucleotide position 132. This mutation caused the conversion of a non-polar, hydrophobic Tryptophan (TGG) into a stop codon (TGA) at the amino acid position 44. The W44X mutation produces a significantly shorter Cx26 protein that is unable to function correctly as the mutation lies within the first extracellular loop.

The W44X variant is classified on the Connexin Homepage (2003) as a disease-causing mutation that leads to non-syndromic autosomal recessive hearing impairment. The W44X mutation was reported in a study involving an American population at a frequency of 0.017 amongst those that suffered from non-syndromic autosomal recessive deafness (Prasad *et al.*, 2000). Refer to Section 4.5.1 for further

information concerning the statistical analysis of the W44X mutation with regards to the frequencies that this variation was detected within this study.

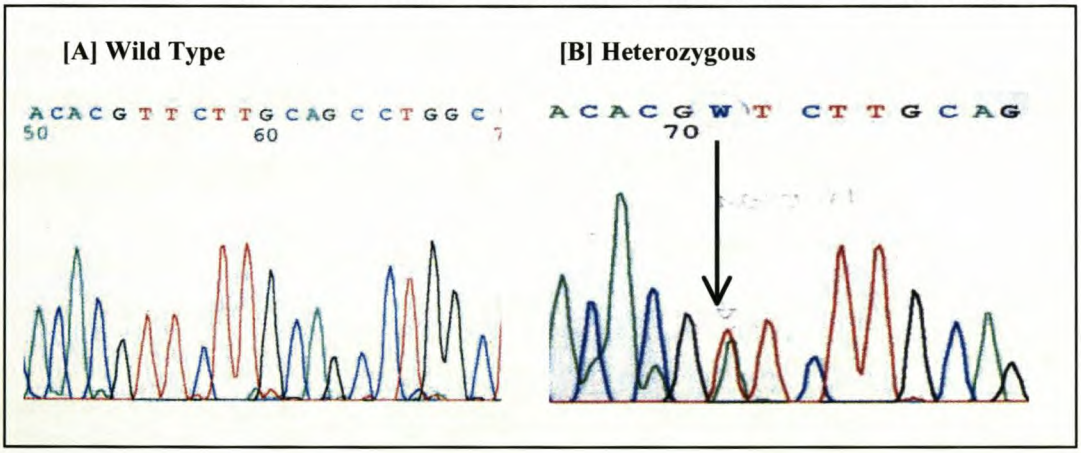
Figure 4.13 A - B: Representative electropherograms of the* GJB2 *mutation W44X



4.4.3.7 THE N62I MUTATION

The N62I mutation of *GJB2* was only detected in a single Caucasian family from the familial patient group of samples (Section 4.5.1). This mutation was observed in compound heterozygous form with the *GJB2* 35delG mutation (Figure 4.14B).

Figure 4.14 A - B: Representative electropherograms of the GJB2 mutation N62I



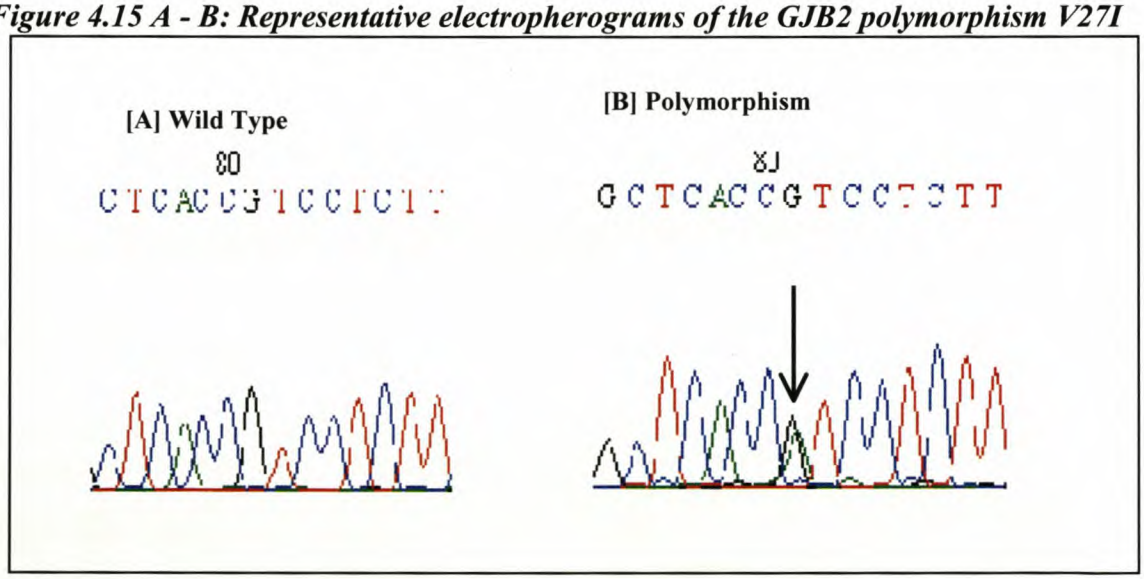
The N62I missense mutation that was detected in *GJB2* was a novel mutation and was the result of the conversion of an A to a T at nucleotide position 185. This mutation causes the change from a polar,

hydrophilic Asparagine (AAC) to a non-polar, hydrophobic Isoleucine (ATC) at amino acid position 62. It was determined that the N62I variation was a disease causing mutation that results in non-syndromic autosomal recessive hearing impairment because it was the only variation, other than 35delG, that was detected in the individual with non-syndromic autosomal recessive deafness. The N62I mutation likely alters the shape of the first extracellular loop due to the difference in size of the amino acids. The change will also likely affect the correct functioning of this domain due to the difference in chemical properties of these amino acids.

4.4.3.8 THE V27I POLYMORPHISM

The V27I polymorphism of *GJB2* was detected in two individuals. These individuals were identified in the Caucasian and Mixed Ancestry control groups (Section 4.5.2). The electropherogram of this polymorphism is shown in Figure 4.15B.

Figure 4.15 A - B: Representative electropherograms of the GJB2 polymorphism V27I



The V27I benign polymorphism that was detected in *GJB2* was the result of the conversion of a G to an A at nucleotide position 79. This polymorphism resulted from the conversion of a non-polar, hydrophobic Valine (GTC) to a non-polar, hydrophobic Isoleucine (ATC) at the amino acid position 27. The Valine amino acid residue lies in the first transmembrane domain of the protein and is found to be conserved in all connexin proteins (Kelley *et al.*, 1998). Even though the amino acid at this position is conserved it is unlikely to play an important role in the correct functioning or construction of the Cx26 protein since individuals that have normal hearing and are homozygotic for the V27I variant have

been identified (Wilcox *et al.*, 2000). However, it has been observed that when the V27I polymorphism is present together with a second *GJB2* polymorphism, E114G, the resultant autosomal non-syndromic recessive deafness phenotype does present. It has been suggested that V27I together with E114G produces a dominant negative effect possibly due to interfering with the function of wild type Cx26 (Park *et al.*, 2000).

From the time that the V27I variant was reported it has been classified as a benign polymorphism (Kelley *et al.*, 1998). Since then extensive evidence has arisen to support this classification of the V27I variant. This evidence was generated in the form of results from a number of studies that were conducted worldwide, where the V27I variant was found in the heterozygotic state as well as the homozygotic form in many individuals with normal hearing (Kenna *et al.*, 2001).

The frequency of the V27I variant has been determined in a number of populations, including the Japanese, Korean, American, French and Iranian (Table 4.4). The V27I polymorphism appears to be very common in the Japanese and Korean populations therefore, suggesting a common ancestor. However, these high frequencies could be inaccurate due to some of the studies using relatively small sample groups. Refer to Section 4.5.2 for further information concerning the statistical analysis of the V27I polymorphism with regard to the frequencies determined for this variation within this study.

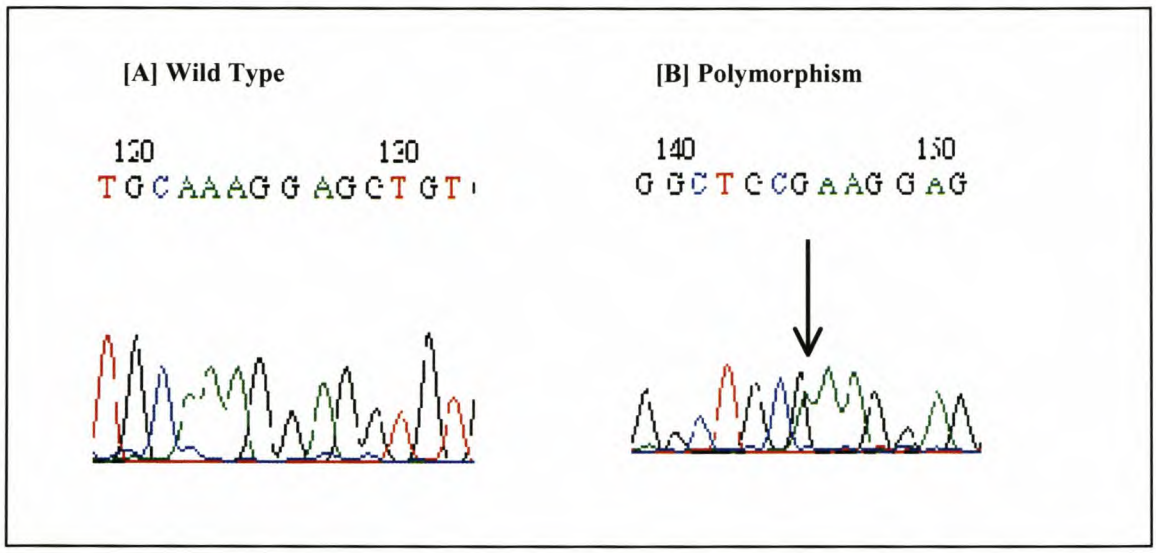
Table 4.4: Frequency of the V27I allele of* GJB2 *in various populations

Population	**V27I Frequency (Deaf Individuals)**	**V27I Frequency (Control Individuals)**	**Reference**
Japanese	------	0.390	Abe *et al.*, 2000
Japanese	------	0.360	Kudo *et al.*, 2000
Korean	------	0.200	Park *et al.*, 2000
American	0.025	------	Prasad *et al.*, 2000
American	0.158	------	Lin *et al.*, 2001
French	------	0.017	Marlin *et al.*, 2001
Iranian	------	0.012	Najmabadi *et al.*, 2002

4.4.3.9 THE A40A POLYMORPHISM

The A40A polymorphism of *GJB2* was only encountered in a single individual from the Mixed Ancestry control group (Section 4.5.2). The electropherogram of this polymorphism is shown in Figure 4.16B.

Figure 4.16 A - B: Representative electropherograms of the* GJB2 *polymorphism A40A



The A40A polymorphism that was detected in *GJB2* was the result of the conversion of an A to a G at nucleotide position 120. This polymorphism lead to the wobble effect occurring, where there is no change in amino acid, as the amino acid at position 40 remains a non-polar, hydrophobic Alanine and only the translation codon changes from GCA to GCG. This change therefore, has no effect on the correct functioning or construction of the Cx26 protein even though the A40A polymorphism lies in the first extracellular loop.

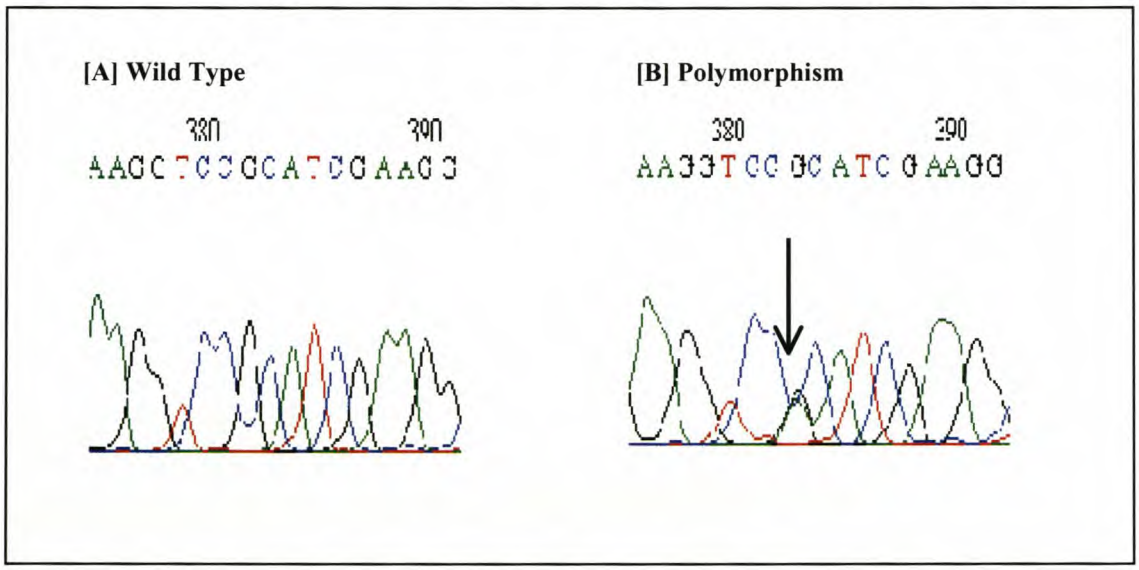
4.4.3.10 THE R127H POLYMORPHISM

The R127H polymorphism of *GJB2* was encountered in three unrelated individuals, all of whom belonged to the Mixed Ancestry control group (Section 4.5.2). The electropherogram of this polymorphism is shown in Figure 4.17B.

The R127H polymorphism that was detected in *GJB2* was the result of the conversion of a G to an A at nucleotide position 380. This polymorphism lead to the conversion of a positively charged, polar

Arginine (CGC) to another positively charged, polar Histidine (CAC) at amino acid position 127. It has been noted that the Arginine residue at codon position 127 is conserved in the B2, A2, A3, A4 and A8 connexins of all species that have been studied to date, however, a histidine residue has been found in other members, such as A1, A5 and B1 (Estivill *et al.*, 1998). The R127H polymorphism occurred in the cytoplasmic loop, which plays a role in pH gating of the Cx26 protein (Rabionet *et al.*, 2000). However, this change had no effect on the correct functioning or construction of the Cx26 protein possibly due to both amino acids being similar in chemical properties and size.

Figure 4.17 A - B: Representative electropherograms of the GJB2 polymorphism R127H



At first it was not known what role the R127H variant played in non-syndromic autosomal recessive hearing impairment as it was restricted to patients suffering from deafness in Spanish, Italian and Greek populations. Therefore, it could not be determined if this variation was a disease-causing mutation or a benign polymorphism (Estivill *et al.*, 1998 and Antoniadi *et al.*, 2000). Only when the R127H variant was detected together with the 35delG mutation in a normal-hearing individual in France, was the classification of the variant as a polymorphism made (Marlin *et al.*, 2001).

The frequency of the R127H variant has since been determined in a number of populations, including the Spanish, Italian, Geek, American, French and Iranian (Table 4.5). The R127H polymorphism appears to be common in the Greek and Iranian populations. However, these high frequencies could be due to the studies using relatively small sample groups. Refer to Section 4.5.2 for further information

concerning the statistical analysis of the R127H polymorphism with regards to the frequencies with which this variation was detected within this study.

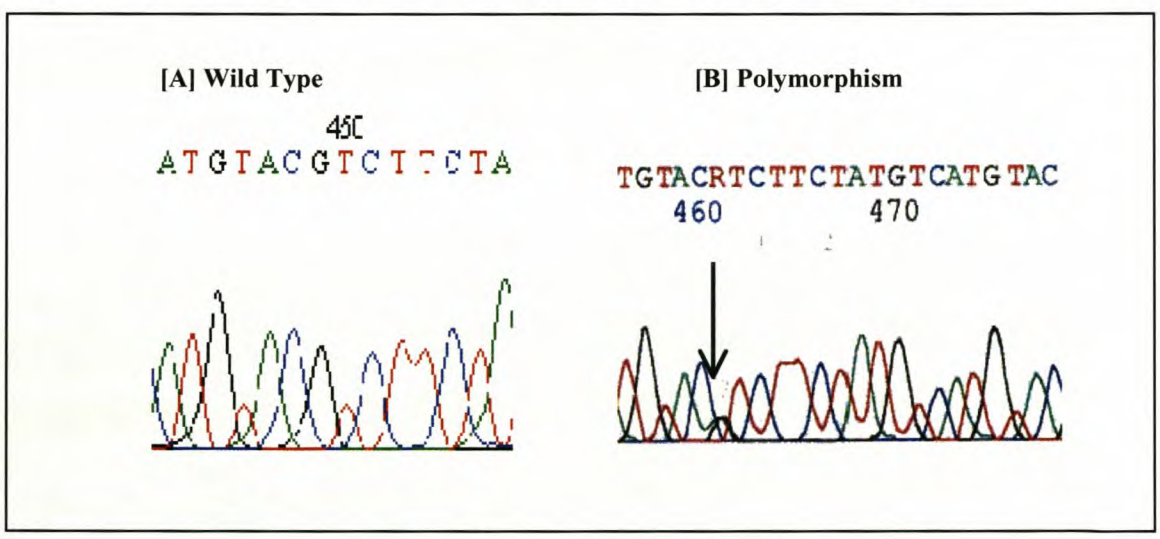
Table 4.5: Frequency of the R127H allele of* GJB2 *in various populations

Population	R127H Frequency (Deaf Individuals)	R127H Frequency (Control Individuals)	Reference
Spanish/Italian	0.012	0.0	Estivill *et al.*, 1998
Greek	0.038	------	Antoniadi *et al.*, 2000
American	0.008	------	Prasad *et al.*, 2000
French	0.010	------	Marlin *et al.*, 2001
Iranian	------	0.024	Najmabadi *et al.*, 2002

4.4.3.11 THE V153I POLYMORPHISM

The V153I polymorphism of *GJB2* was identified in one Caucasian and one Indian family from the sporadic patient group. The electropherogram of this polymorphism is shown in Figure 4.18B.

Figure 4.18 A - B: Representative electropherograms of the GJB2 polymorphism V153I



The V153I polymorphism that was detected in *GJB2* was the result of the conversion of a G to an A at nucleotide position 457. This polymorphism resulted in the change from a non-polar, hydrophobic Valine (GTC) to another non-polar, hydrophobic Isoleucine (ATC) at the amino acid position 153. This polymorphism occurred in the third transmembrane domain of the Cx26 protein. However, this change

had no effect on the correct functioning or construction of the Cx26 protein possibly due to both amino acids being similar in chemical properties and size.

At first it was unclear as to the role that the V153I variant played in non-syndromic autosomal recessive deafness since it had only been identified in heterozygotic form amongst individuals suffering from hearing loss (Prasad *et al.*, 2000 and Kenna *et al.*, 2001). Later, two individuals with normal hearing were identified with the V153I variant together with the 35delG mutation of *GJB2*. Therefore, the V153I variant was classified as a benign polymorphism with no significant no role in non-syndromic austosomal recessive deafness (Marlin *et al.*, 2001).

Table 4.6: Frequency of the V153I allele of* GJB2 *in various populations

Population	V153I Frequency (Deaf Individuals)	V153I Frequency (Control Individuals)	Reference
American	0.025	------	Prasad *et al.*, 2000
American	------	0.011	Kenna *et al.*, 2001
French	------	0.021	Marlin *et al.*, 2001
Iranian	------	0.060	Najmabadi *et al.*, 2002

The frequency of the V153I variant has been determined in a few populations, including the American, French and Iranian (Table 4.6). The V153I polymorphism appears to be fairly common in all these populations, especially the Iranian population. However, these high frequencies that have been reported could be due to the studies using relatively small sample groups. Refer to Section 4.5.2 for further information concerning the statistical analysis of the V153I polymorphism with regards to the frequencies with which this variation was detected within this study.

4.4.4 SCREENING OF *GJB6*

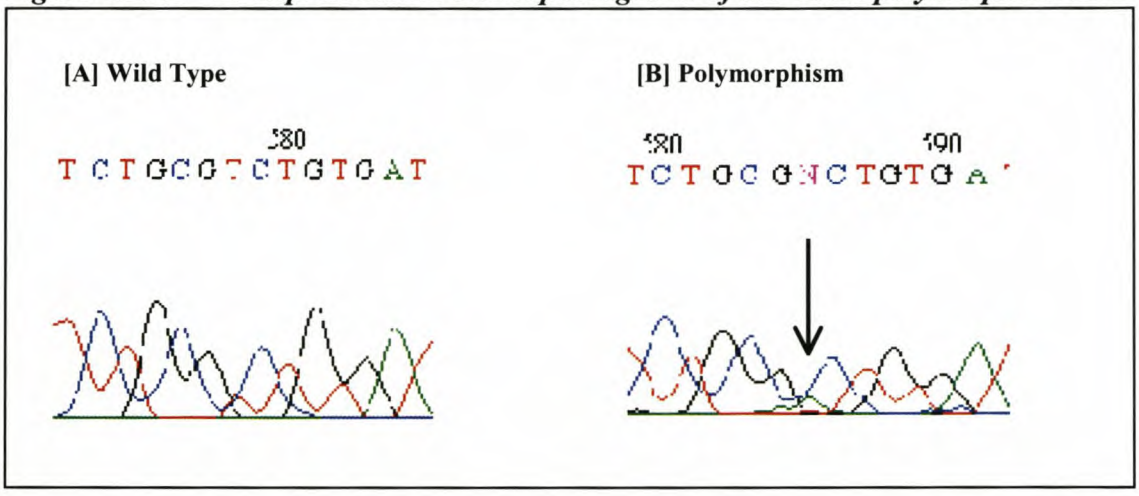
A number of samples that were screened for Cx26 (*GJB2*) mutations exhibit none or only one disease causing mutation and it was decided to screen these samples for mutations in *GJB6*. The Cx30 gene was chosen because of the role it plays together with *GJB2* in hearing. Another reason for choosing the *GJB6* gene was that mutations within this gene have been implicated in non-syndromic autosomal recessive deafness (especially the large deletion, Δ(*GJB6*-D13S1830) mutation). To screen *GJB6*, automated DNA sequencing was selected because it is the most effective and sensitive method used to

detect mutations. Another advantage of using DNA sequencing is that the coding region of the *GJB6* gene is relatively small (786 bp) and therefore mutation screening can be accomplished in a single DNA sequencing reaction. To detect the Δ(*GJB6*-D13S1830) mutation, PCR amplification involving specific primers followed by agarose gel electrophoresis was used.

4.4.4.1 THE S199T POLYMORPHISM

The only variation identified by DNA sequencing, was the S199T polymorphism, which was encountered in a single individual of the Mixed Ancestry control group (Section 4.5.2). The electropherogram of this polymorphism is shown in Figure 4.19A -B.

Figure 4.19 A - B: Representative electropherograms of the* GJB6 *polymorphism S199T



The S199T polymorphism that was detected in *GJB6* was the result of the conversion of a T to an A at nucleotide position 595. This polymorphism resulted from the conversion of a polar, hydrophilic Serine (TCT) to another polar, hydrophilic Threonine (ACT) at the amino acid position 199. This polymorphism occurred in the fourth transmembrane domain of the Cx30 protein. However, this change had no effect on the correct functioning or construction of the Cx30 protein possibly due to both amino acids being similar in chemical properties and size.

4.4.4.2 THE Δ(*GJB6*-D13S1830) MUTATION

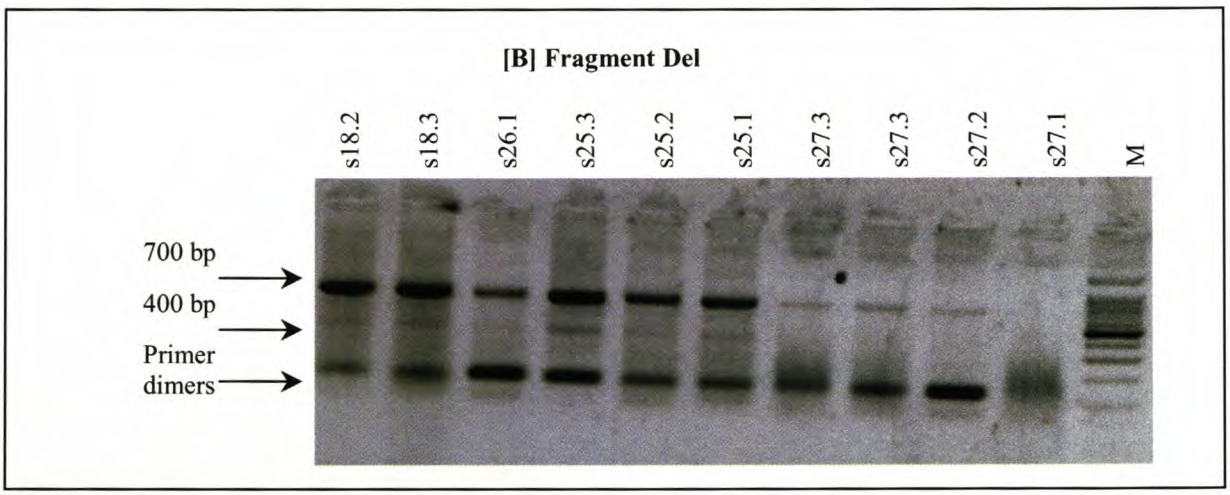
There have been three reports concerning a large deletion in *GJB6* (Lerer *et al.*, 2001; Pallares-Ruiz *et al.*, 2002; Del Castillo *et al.*, 2002). The first study reported a deletion of at least 140 kb that was identified in seven patients from four unrelated Ashkenazi Jewish families (Lerer *et al.*, 2001). It was noted that the deletion acted *in trans* with *GJB2* mutations, 35delG and 167delT. It was suggested that the deletion caused non-syndromic autosomal recessive deafness either by a digenic mode of inheritance that involved *GJB2* and *GJB6*, or it removed control elements that were important for the expression of *GJB2*. It was also demonstrated that all the patients had the same haplotypic background, therefore it was assumed that the 140 kb deletion was due to a founder mutation. The frequency of the 140 kb deletion was determined to be less than 0.005 amongst the general population, but relatively common amongst those with non-syndromic autosomal recessive deafness, at a frequency of 0.037 (Lerer *et al.*, 2001).

The second study reported a deletion of 150 kb in five French patients with non-syndromic autosomal recessive hearing loss who had previously been identified as carrying a single *GJB2* mutation or were linked to DFNB1 but no mutations in *GJB2* had been identified (Palleres-Ruiz *et al.*, 2002). It was shown that in these patients the 150 kb deletion was acting *in trans* with the *GJB2* mutations 35delG and E47X. The 150 kb deletion was also observed in homozygotic state. Therefore, it was concluded that the 150 kb deletion, which involves *GJB6*, is a recessive mutation but it was not known whether it was of ancestral or recurrent origin or even a combination of both (Pallares-Ruiz *et al.*, 2002).

Finally, a 342 kb deletion involving *GJB6* was reported in a Spanish and Cuban population (Del Castillo *et al.*, 2002). The 342 kb deletion was identified in homozygous and heterozygous states in patients with non-syndromic autosomal recessive hearing impairment. It was not known whether the 342 kb deletion encompassed the 150 and 140 kb reported deletions since the breakpoints for the smaller deletions had not been reported. The frequency of the 342 kb deletion was determined at 0.048 amongst the Cuban and 0.088 amongst the Spanish non-syndromic autosomal recessive deafness patients. It was not detected in any of the 200 samples collected from individuals with normal hearing (Del Castillo *et al.*, 2002). In a recent study it has been determined that the three large deletions that were identified in various populations overlap and consists of a deletion of approximately 309 kb, known as the Δ(*GJB6*-D13S1830) mutation (Del Castillo *et al.*, 2003).

In the current study, agarose gel electrophoresis was used for the detection of the absence or presence of the Δ(*GJB6*-D13S1830) mutation (Figure 4.20). Theoretically a fragment would only be produced in the presence of the large deletion as it resulted in the primers being within the necessary distance for amplification to occur. An amplicon would not be produced when the large deletion was not present, since the two primers would be too far apart for successful amplification to occur.

Figure 4.20: Representative photograph of PCR amplicons obtained during Δ(GJB6-D13S1830) mutation screening



Agarose gel electrophoresis of the PCR amplified fragment Del that covers the Δ(*GJB6*-D13S1830) mutation. The 2% agarose gels were electrophoresed for 1 hour at 100V in 1X TBE buffer and stained with EtBr. M = 100 bp DNA ladder [Promega Corporation, Madison, WI, USA].

For the Δ(*GJB6*-D13S1830) mutation screening a Del fragment of 460 bp was expected (Del Castillo *et al.*, 2002). However, in the current study a fragment of approximately 700 bp was observed instead. It was therefore, assumed that this fragment could possibly indicate the presence of a smaller deletion of approximately 140 kb. If a fragment of 460 bp was present it would indicate the presence of the larger deletion of 324 kb as the primers are brought closer together than if there was only a 140 kb deletion, thereby producing a 700 bp fragment instead. This 700 bp fragment together with a smaller 400 bp fragment, which was occasionally observed, was purified from the gel and sequenced to determine the breakpoints of the deletion.

During the determination of the breakpoints a number of interesting observations were made. Firstly, it was discovered that when the fragment of approximately 700 bp was compared against the NCBI database, using the BLAST algorithm, a number of matches were obtained throughout the genome. The predominant hit was that with the human DNA sequence from clone RP11-501K3 on chromosome 13 (accession number: AL355984). The fragment was positioned from nucleotide 43 075 – 43 722 on this clone, within the vicinity of *GJB6*. However, it was not expected that over 300 matches would be obtained in both the complementary and reverse complementary orientation. These matches included correspondence with clones from almost all the chromosomes, including the X chromosome, as well as the protein S (alpha) (*PROS1*) gene, part of a novel gene (*IMOGEN 38*), the FSH primary response homolog 1 (*FSHPRH1*) pseudogene, the menage a trios 1 (CAK assembly factor) (*MNAT1*) gene and the glypican 3 (*GPC3*) gene. A possible reason for this result is that the sequence of the 700 bp fragment is that of a genome-wide repeat i.e. a long interspersed nuclear element (LINE). LINEs are classified, together with short interspersed nuclear elements (SINEs), as retroposons since they contain a reverse transcriptase-like gene that possibly plays a role in retro transposition. Retroposons and LTR elements, which contain long terminal repeats at either end that are involved in transposition, are known as retro elements. These retro elements are known features of eukaryotic genomes. These sequences are found often throughout the genome, as is seen in the example of LINE-1 that is 6.1 kb in length and is found 3 500 times within the human genome (Brown, 1999).

Secondly, when the second fragment of approximately 400 bp was compared against the NCBI database it was reported to have a match with the human DNA sequence from clone RP5-1033H22 on chromosome 1p21.2-22.2 (accession number: AL109613) was obtained. This clone contains part of a novel gene (KIAA0554) and the *BCAR3* (breast cancer anti-estrogen resistance 3) gene. The 330 bp fragment was located at nucleotide position 39 047 – 39 277 within this clone. It is unclear as to why this fragment was produced but it could possibly be due to the sequence being similar to enough to the Δ(*GJB6*-D13S1830) sequence, which allows for the non-specific binding of the primers used during PCR amplification.

Due to the result obtained from the NCBI database analysis of the 700 bp and 400 bp fragments it was decided to perform a homology search with the sequences of the primers used to amplify these fragments. Again a number of interesting observations were made.

The results of the NCBI database analysis of the forward primer, GJB6-1R, indicated that the sequence of this primer had homology with three locations within the human genome: Firstly, as expected, in the complementary orientation within *GJB6* (accession number: NM_006783) at nucleotide position 687 – 710 of the sequence of this gene. Secondly, the sequence matched, in the forward orientation, to the THYRO1000678 clone (accession number: AK075247) at nucleotide position 1 140 – 1 163. This is not unexpected as this clone is that of a *Homo sapiens* cDNA sequence that is highly similar to the *GJB6* gene. Finally, the sequence was also found in the reverse complementary orientation within the RP11-501K3 clone on chromosome 13 at nucleotide position 20 612 – 20 635. This could possibly explain why a 700 bp fragment was obtained instead of the expected 460 bp. The reason could be that the GJB6-1R primer acts as both a forward and reverse primer to produce the 700 bp fragment in the presence of the Δ(*GJB6*-D13S1830) mutation since the primers are in closer proximity to one another. The 460 bp fragment would be the result of amplification between the correct primers, GJB6-1R and BKR-1, when the Δ(*GJB6*-D13S1830) mutation is present.

When the reverse primer BKR-1 was analyzed using the NCBI database it was again noted that the primer matched a number of sequences within the human genome both in the forward and reverse complement orientation. This primer matched predominantly with the RP11-476H16 clone on chromosome 13 (accession number: AL590096). The primer was found to be located in the reverse complementary orientation at nucleotide position 62 369 – 62 393. This result was expected as the clone is situated close to *GJB6*. However, it was not expected that the primer would also match sequences found in two other clones. Firstly, the primer was found to show 92% similarity with the forward DNA sequence of clone RP11-11K15 on chromosome 5 (accession number: AC091888) at nucleotide position 9 0154 – 9 0178. Secondly, the primer showed a 92% match at nucleotide position 3 3704 – 3 3728 in the reverse orientation to the sequence of clone RP3-522O2 on chromosome 6q21 (accession number: HS522O2). It is not thought that these matches have an influence on the sensitivity of the PCR amplification as they are not located on chromosome 13 and they are not completely complementary to the genomic sequence.

Due to the information discovered during the NCBI analysis of the PCR fragments and primers raising questions as to the accuracy of the screening method, it was decided not to include the result obtained from the screening for the Δ(*GJB6*-D13S1830) mutation. Another reason for excluding the results was the latest report by Del Castillo *et al* (2003) that only the Δ(*GJB6*-D13S1830) mutation of

approximately 309 kb exists and not any of 140 or 150 kb. A final reason for the exclusion of the results was that there was no reproducibility of results for the Δ(*GJB6*-D13S1830) mutation as there was inconsistent PCR amplification.

4.4.5 INACCURACIES IN THE NCBI DATABASE

During the analysis of the sequences of *GJB2* and *GJB6* a few inaccuracies within the NCBI database sequences were noted. This was not expected, since the sequences in the NCBI database are those from Caucasian samples and in this study many Caucasians were included. The first variation was detected when the *GJB2* sequence (accession number: NM_004004) on the NCBI database was compared with the sequences that were obtained from the screening of the samples from patients and controls. It was noted within all these samples that they contained the S86T variation, which involves the inversion of a GC to a CG at nucleotide position 257 and 258, which leads to the amino acid conversion of a serine to a threonine. Therefore, it was assumed that the NCBI database sequence represents a rare variant and not the wild type.

The second inaccuracy within the NCBI database was detected in the *GJB6* sequence (accession number: NM_006783). The L36L variant was observed in all the patient and control samples that were screened for *GJB6* mutations. This variation involves the conversion of an A to a C at nucleotide position 108, which results in no change in amino acid as both variants lead to the formation of a leucine at this position. The third inaccuracy was also observed in the *GJB6* sequence as the H124Q variant. This change in the sequence of a C to a G at nucleotide position 372 results in the amino acid conversion of a histidine to a glutamine. It was therefore assumed that the sequence on the NCBI database does not represent the wild type allele but rather a rare variant.

4.5 THE STATISTICAL ANALYSIS OF DATA

The statistical analysis of the data collected during this research project included the determination of the allele frequencies of the specific disease-causing mutations and benign polymorphisms that were detected in both *GJB2* and *GJB6* for the different population groups that were studied. Also included in

the statistical analysis was the determination of the success rate achieved in identifying the causative mutations. A summary of the results of the molecular analysis of *GJB2* and *GJB6* is presented C.

4.5.1 ALLELE FREQUENCIES OF THE DISEASE-CAUSING MUTATIONS DETECTED IN *GJB2* AND *GJB6*

4.5.1.1 FAMILIAL GROUP ALLELE FREQUENCIES

Allele frequencies were determined for the mutations in *GJB2* within the familial study group. This was achieved by using the mutation analysis results of the proband for each of the families.

Table 4.7 displays the number of alleles observed for each of the *GJB2* mutations for each of the specific families that were classified as familial cases. The 35delG mutation of *GJB2* was the most common mutation within this study group, with an allele frequency of 0.281 (of 32 alleles). The *GJB2* mutation 312del14 was also detected at a high frequency of 0.188 within this study group. The only other mutation that was identified amongst the familial cases was the novel N62I mutation of *GJB2*, which was found in a single family.

Table 4.7: Allele frequencies of the mutations detected in* GJB2 *in the familial study cohort of 16 families

Family Number	Number of Alleles						
	35delG	312del14	W24X	M34T	V37I	W44X	N62I
1	0	0	0	0	0	0	0
2	1	0	0	0	0	0	1
3	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0
5	1	1	0	0	0	0	0
6	2	0	0	0	0	0	0
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	2	0	0	0	0	0	0
10	0	2	0	0	0	0	0
11	1	1	0	0	0	0	0
12	2	0	0	0	0	0	0
13	0	0	0	0	0	0	0
14	0	2	0	0	0	0	0
15	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0
Total	9	6	0	0	0	0	1
Frequency	0.281	0.188	0.0	0.0	0.0	0.0	0.031

4.5.1.2 SPORADIC GROUP ALLELE FREQUENCIES

The allele frequencies of the mutations that were detected in *GJB2* were also calculated for the sporadic study group (Table 4.8). The calculation was achieved by using the mutation analysis results obtained for the proband of each of the families.

Table 4.8: Allele frequencies of the mutations detected in* GJB2 *in the sporadic cohort of 28 families

Family Number	Number of Alleles						
	35delG	312del14	W24X	M34T	V37I	W44X	N62I
1	1	0	0	0	0	0	0
2	1	1	0	0	0	0	0
3	1	1	0	0	0	0	0
4	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0
7	0	1	0	0	0	0	0
9	1	0	1	0	0	0	0
10	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
12	0	1	0	0	0	0	0
13	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0
17	2	0	0	0	0	0	0
18	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0
20	0	2	0	0	0	0	0
21	0	0	0	0	1	0	0
22	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0
26	0	1	0	0	0	0	0
27	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0
Total	6	7	1	0	1	0	0
Frequency	0.107	0.125	0.018	0.0	0.018	0.0	0.0

The *GJB2* mutations 35delG and 312del14 were found at high frequencies of 0.107 and 0.125 respectively, with the 312del14 being the most common mutation amongst this group. The total number of alleles screened was 56. The only other mutations observed were W24X and V37I. Both these mutations were only observed in single families, and therefore at a very low frequency.

When comparing the sporadic group (Table 4.8) to the familial group (Table 4.7) it can be noted that the 140 kb deletion of *GJB6* was more common amongst the sporadic cases whereas the 35delG

mutation was more common in the familial group. The *GJB2* mutation 312del14 was found at relatively the same frequency amongst both groups. It appears as if there were more variation in the type of mutations amongst the sporadic cases since a missense (V37I) and a nonsense (W24X) mutation was detected in this group whereas, in the familial group only a single missense (N62I) mutation was observed.

4.5.1.3 TOTAL ALLELE FREQUENCIES

When the total allele frequencies (Table 4.9) for the mutations observed involving *GJB2* were calculated, using all the groups studied in this project, it was observed that the most common mutation was the 35delG mutation with a frequency of 0.052, which was followed by the 312del14 at a frequency of 0.049. This was calculated from a total of 288 alleles that were screened during this study. The remaining *GJB2* mutations were encountered at relatively low frequencies and are therefore apparently not common to the South African population

Table 4.9: Allele frequencies of the mutations detected in* GJB2 *in the entire study group

Study Group	Number of Alleles						
	35delG	312del14	W24X	M34T	V37I	W44X	N62I
Familial	9/32 (0.281)	6/32 (0.188)	0/32 (0.0)	0/32 (0.0)	0/32 (0.0)	0/32 (0.0)	1/32 (0.031)
Sporadic	6/56 (0.107)	7/56 (0.125)	1/56 (0.018)	0/46 (0.0)	1/56 (0.018)	0/56 (0.0)	0/56 (0.0)
Caucasian Controls	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)
Mixed Ancestry Controls	0/100 (0.0)	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)
Total (n=288)	15	14	2	1	1	1	1
Frequency	0.052	0.049	0.007	0.003	0.003	0.003	0.003

4.5.1.4 POPULATION-BASED ALLELE FREQUENCIES

The allele frequencies for each of the mutations that were detected in *GJB2* were also determined for the Caucasian and Mixed Ancestry population groups of South Africa. The results that were calculated for the Caucasian population group are indicated in Table 4.10.

From a total of 164 alleles that was screened it was determined that the 312del14 and 33delG mutations were the most common with frequencies of 0.085 and 0.073, respectively. The W44X, M34T and N62I mutations were observed at very low frequencies. The remaining two mutations W24X and V37I were not present in this population group. Therefore, these variations are apparently uncommon specifically in the South African Caucasian population.

Table 4.10: Allele frequencies of the mutations detected in* GJB2 *in the South African Caucasian population

Study Group	Number of Alleles						
	35delG	312del14	W24X	M34T	V37I	W44X	N62I
Familial	7/28 (0.250)	6/28 (0.214)	0/28 (0.0)	0/28 (0.0)	0/28 (0.0)	0/28 (0.0)	1/28 (0.036)
Sporadic	5/36 (0.139)	7/36 (0.194)	0/36 (0.0)	0/36 (0.0)	0/36 (0.0)	0/36 (0.0)	0/36 (0.0)
Caucasian Controls	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)	1/100 (0.010)	0/100 (0.0)
Total (n=164)	12	14	0	1	0	1	1
Frequency	0.073	0.085	0.0	0.006	0.0	0.006	0.006

The total allele frequencies for the mutations observed in *GJB2* also calculated for the Mixed Ancestry population of South Africa (Table 4.11). The most common mutation detected at a frequency of 0.027 (112 alleles) was the 35delG mutation. The only other mutation that was detected amongst the Mixed Ancestry population was the W24X variation, which was found at a low frequency of 0.018. Interestingly, the *GJB2* mutation 312del14 that was observed amongst the Caucasian South African population (0.085) was not detected at all amongst the Mixed Ancestry population of South Africa.

Table 4.11: Allele frequencies of the mutations detected in* GJB2 *in the South African Mixed Ancestry population

Study Group	Number of Alleles						
	35delG	312del14	W24X	M34T	V37I	W44X	N62I
Familial	2/4 (0.500)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
Sporadic	1/8 (0.125)	0/5 (0.0)	1/8 (0.125)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
Mixed Ancestry Controls	0/100 (0.0)	0/100 (0.0)	1/100 (0.001)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)
Total (n=112)	3	0	2	0	0	0	0
Frequency	0.027	0.0	0.018	0.0	0.0	0.0	0.0

The allele frequencies for all the mutations that were detected in *GJB2* compared with the frequencies that have been previously reported. The results of this are discussed briefly below:

- For the *GJB2* mutation 35delG it was noted that the allele frequency obtained in the Caucasian population (0.073) in this study was higher than any that had been previously reported (Table 2.5), however, this could be the result of using a relatively small study group. The allele frequency that was obtained for the 35delG mutation for the Mixed Ancestry population (0.027) was similar to those that had been reported in the general population of countries such as Belgium, France and USA (Americans of European ancestry).
- The 312del14 mutation of *GJB2* was only detected in the Caucasian population in this study at a frequency of 0.085. This frequency appears to be relatively high compared with the previously reported frequency of 0.026 in an American population with non-syndromic autosomal recessive deafness (Lin *et al.*, 2001). Therefore, it appears that the 312del14 mutation is possibly specific to the South African Caucasian population, however it must be taken into consideration that a relatively small sample size was used in this study. Refer to Section 4.4.3.2 for more information concerning the 312del14 mutation.
- The *GJB2* mutation W24X was only detected in the Mixed Ancestry population (0.018). When compared with previously reported cases, this result is not surprising as it has been observed that the W24X mutation is not frequently found but it is predominantly detected amongst individuals from the countries of India, Pakistan and Bangladesh (Rickard *et al.*, 2001). Refer to section 4.4.3.3 for more information concerning the W24X mutation.

- The M34T mutation of *GJB2* was only detected in the Caucasian population at a frequency of 0.006, which is similar to that reported in a French population. This frequency is relatively low compared with some of the other reported frequencies in populations such as the British/Irish, Australian and American (Table 4.2).
- The *GJB2* mutation V37I was not detected in either the Caucasian or Mixed Ancestry populations of South Africa however, it was detected in a single family of Indian ancestry. This was surprising since the V37I mutation has been reported at relatively high frequencies amongst various other populations such as the Japanese, American, Australian and French (Table 4.3). A possible reason for the low number of V37I mutants detected in this study could be due to the sample size.
- The nonsense mutation W44X of *GJB2* was only detected at a frequency of 0.006 amongst the South African Caucasian population in this study, which is similar to the previously reported frequency of 0.017 that was observed in an American group with non-syndromic autosomal recessive deafness (Prasad *et al.*, 2000). Refer to Section 4.4.3.6 for further information concerning the W44X mutation.
- The N62I mutation of *GJB2* that was detected in a single Caucasian family was a novel mutation and therefore, there are no previously reported frequencies to compare this mutation to.

4.5.2 ALLELE FREQUENCIES OF BENIGN POLYMORPHISMS DETECTED IN *GJB2* AND *GJB6*

4.5.2.1 FAMILIAL GROUP ALLELE FREQUENCIES

The allele frequencies of the polymorphisms that were detected in *GJB2* and *GJB6* were also determined for the different groups studied in this research project. There were no polymorphisms detected amongst familial cases but this is possibly due to the small sample size screened.

4.5.2.2 SPORADIC GROUP ALLELE FREQUENCIES

The results obtained for the calculation of the allele frequencies for the polymorphisms that were detected in *GJB2* and *GJB6* amongst the sporadic study group are indicated in Table 4.12. Again only a

single variation was detected in this study group; *GJB2* polymorphism V153I that was observed at a high frequency of 0.071 amongst a total of 56 alleles. This was a small population size therefore, the frequency has a high probability of being inacurrately estimated.

Table 4.12: Allele frequencies of the polymorphisms detected in* GJB2 *and* GJB6 *in the sporadic study cohort of 28 families

Family Number	Number of Alleles				
	GJB2				*GJB6*
	V27I	A40A	R127H	V153I	S199T
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	2	0
11	0	0	0	0	0
12	0	0	0	2	0
13	0	0	0	0	0
14	0	0	0	0	0
15	0	0	0	0	0
16	0	0	0	0	0
17	0	0	0	0	0
18	0	0	0	0	0
19	0	0	0	0	0
20	0	0	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
26	0	0	0	0	0
26	0	0	0	0	0
27	0	0	0	0	0
28	0	0	0	0	0
29	0	0	0	0	0
Total	0	0	0	4	0
Frequency	0.0	0.0	0.0	0.071	0.0

4.5.2.3 TOTAL ALLELE FREQUENCIES

The total allele frequencies of the polymorphisms that were detected in *GJB2* and *GJB6* were calculated (Table 4.13). The combining of all the groups studied, increased the number of alleles screened to a total of 276. It was observed that *GJB2* polymorphisms V27I and V153I were the most common at a frequency of 0.015 each. The second most common variation was R127H of *GJB2*. The remaining polymorphisms were observed at a lower frequency of 0.003 and therefore are assumed to be uncommon to the South African population.

Table 4.13: Allele frequencies of the polymorphisms detected in* GJB2 *and* GJB6 *in the entire study group

Study Group	Number of Alleles				
	GJB2				*GJB6*
	V27I	A40A	R127H	V153I	S199T
Familial	0/32 (0.0)	0/32 (0.0)	0/32 (0.0)	0/32 (0.0)	0/32 (0.0)
Sporadic	0/56 (0.0)	0/56 (0.0)	0/56 (0.0)	4/56 (0.091)	0/56 (0.0)
Caucasian Controls	2/100 (0.02)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)
Mixed Ancestry Controls	2/100 (0.02)	2/100 (0.02)	6/100 (0.06)	0/100 (0.0)	2/100 (0.02)
Total (n=288)	4	2	6	4	2
Frequency	0.014	0.007	0.021	0.014	0.007

4.5.2.4 POPULATION-BASED ALLELE FREQUENCIES

The allele frequencies were also calculated for each of the population groups studied to determine if any polymorphism involving *GJB2* or *GJB6* are specific to that group. The results of determination of the allele frequency for the Caucasian population of South Africa are indicated in Table 4.14. It was observed that no single polymorphism was specific to this group however two *GJB2* variations were detected, which included V27I and V153I, at a frequency of 0.012 and 0.006 respectively from 164 alleles screened.

Table 4.14: Allele frequencies of the polymorphisms detected in* GJB2 *and* GJB6 *in the South African Caucasian population

Study Group	Number of Alleles				
	GJB2				*GJB6*
	V27I	A40A	R127H	V153I	S199T
Familial	0/28 (0.0)	0/28 (0.0)	0/28 (0.0)	0/28 (0.0)	0/28 (0.0)
Sporadic	0/36 (0.0)	0/36 (0.0)	0/36 (0.0)	1/36 (0.028)	0/36 (0.0)
Caucasian Controls	2/100 (0.020)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)	0/100 (0.0)
Total (n=164)	2	0	0	1	0
Frequency	0.0122	0.0	0.0	0.006	0.0

The allele frequencies of the polymorphisms observed in *GJB2* and *GJB6* were also determined for the Mixed Ancestry population of South Africa as is indicated in Table 4.15. In this group a *GJB2* polymorphism, R127H, does appear to be specific as it was detected at a frequency of 5.357% amongst a total of 112 alleles screened. Other variations were observed at a low frequency of 1.786%. These polymorphisms included the *GJB2* variations V27I and A40A as well as the *GJB6* polymorphism S199T. The V153I polymorphism of *GJB2* was not detected amongst the Mixed Ancestry population of South Africa.

Table 4.15: Allele frequencies of the polymorphisms detected in* GJB2 *and* GJB6 *in the South African Mixed Ancestry population

Study Group	Number of Alleles				
	GJB2				*GJB6*
	V27I	A40A	R127H	V153I	S199T
Familial	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
Sporadic	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
Mixed Ancestry Controls	2/100 (0.02)	2/100 (0.02)	6/100 (0.06)	0/100 (0.0)	2/100 (0.02)
Total (n=112)	2	2	6	0	2
Frequency	0.018	0.018	0.054	0.0	0.018

The allele frequencies for all the polymorphisms that were detected in *GJB2* and *GJB6* were compared with the frequencies that have been previously reported. The results of this are discussed briefly below:

- The allele frequency of the *GJB2* variation V27I was similar in both the Caucasian and Mixed Ancestry population groups at a frequency of 0.012 and 0.018 respectively. When compared with previous reports it was noticed that the Caucasian group had a similar frequency to that which has been detected in the Iranian population whereas the Mixed Ancestry population is similar to that which was determined in a French study. The V27I polymorphism has been detected at very high frequencies amongst the Japanese and Korean and it has been assumed that this polymorphism is a founder variation for the Asian populations (Table 4.4).
- The A40A polymorphism of *GJB2* was only detected in a single individual from the Mixed Ancestry control group. It was not possible to find any information concerning the frequency of this variation therefore the allele frequency that was determined in this study could not be compared with previous reports.
- The R127H polymorphism of *GJB2* was not detected in the Caucasian population however it was observed at a relatively high frequency amongst the Mixed Ancestry population of South Africa. The frequency of 0.054 was also relatively high when compared with the frequencies reported in various other studies involving a number of populations. The only similar frequency was that of 0.038 which was detected in a Greek study (Table 4.5). The frequency that was observed in this study could be inaccurate, as the sample size was relatively small.
- The *GJB2* polymorphism of V153I was only detected in the South African Caucasian population and not in the Mixed Ancestry population in this study. It was observed at a relatively low frequency of 0.006, which is lower than any of the other previously reported frequencies determined using a number of different populations (Table 4.6). The frequency for V153I is possibly inaccurate as the sample size used in this study was relatively small.
- The S199T polymorphism of *GJB6* was only detected in the Mixed Ancestry population of South Africa at a frequency of 0.018. It was not possible to find any information concerning the frequency of this variation, therefore the allele frequency that was determined in this study could not be compared with previous reports.

4.5.3 SUCCESS RATE OF MUTATION DETECTION

The success rate of detecting mutations within *GJB2* and *GJB6* was calculated for both the familial and the sporadic study groups, which contained families with non-syndromic autosomal recessive deafness.

These two study groups were also divided according to population, so that the success rate could also be determined for the various populations (Table 4.16).

Table 4.16: Total success rate of mutation detection within* GJB2 *and* GJB6 *according to population and study group

Population	Number of Alleles Identified						Total
	Familial Study Group			Sporadic Study Group			
	GJB2	*GJB6*	Total	*GJB2*	*GJB6*	Total	
Caucasian	14/28 (0.50)	0/28 (0.0)	14/28 (0.50)	12/36 (0.33)	0/36 (0.0)	12/36 (0.33)	26/64 (0.41)
Mixed Ancestry	2/4 (0.50)	0/4 (0.0)	2/4 (0.50)	2/8 (0.25)	0/8 (0.0)	2/8 (0.25)	4/12 (0.33)
African	0/0 (0.0)	0/0 (0.0)	0/0 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)
Indian	0/0 (0.0)	0/0 (0.0)	0/0 (0.0)	1/6 (0.17)	0/6 (0.0)	1/6 (0.17)	1/6 (0.17)
Total	16/32 (0.50)	0/32 (0.0)	16/32 (0.50)	15/56 (0.27)	0/56 (0.0)	15/56 (0.27)	31/88 (0.35)

From a total of 32 alleles found within the familial group a success rate of 50% was achieved in the detection of the deafness causing mutations within these families. This group contained individuals from the South African Caucasian and Mixed Ancestry population groups, which had a success rate for mutation detection of 50% each. It has been previously noted that when screening for *GJB2* mutations, 10 – 42% of the individuals tested have only one mutation that results in hearing loss (Del Castillo *et al.*, 2002). This figure corresponds to those what was observed in this study (50% of the non-syndromic autosomal recessive deafness causing mutations have not been identified in the familial group).

Amongst those individuals who were classified in the sporadic group a success rate of only 26.8% was achieved from 56 alleles that were screened for mutations that lead to hearing loss. In this group there were individuals from various South African populations including Caucasian, Mixed Ancestry, African and Indian. The best success was achieved amongst the Caucasians of this group as 33% of all deafness mutations were identified. A success rate, for mutation detection during screening, of 25% and 16.7% was obtained respectively for the Mixed Ancestry and Indian populations. For the African

group there were no mutations detected during screening. This however, was expected as it was noted in a previous study involving a Ghanaian population that *GJB2* mutations only contribute to 16% of the cause of non-syndromic autosomal recessive deafness and it was thought that other factors such as the environment played a larger role (Hamelmann *et al.*, 2001). This could also be the reason as to why no mutations were detected in either *GJB2* or *GJB6* for any of the African individuals in this study. The frequency of unidentified mutations is 73.2% amongst the sporadic group studied, which is higher than the expected 10 – 42%.

A total success rate of 35.2% was therefore attained from the 88 alleles that were screened for mutations found within *GJB2* and *GJB6*, which result in non-syndromic autosomal recessive deafness. When comparing the two study groups it was observed that the best results were obtained in the familial group, which had a success rate of 50% compared with the sporadic group's rate of 26.8%. When comparing the different populations it was observed that the best success was obtained in the Caucasian population (40.6%) followed by the Mixed Ancestry (33.3%) and the Indian (16.7%) populations. It is expected that the success rate will increase with the inclusion of an accurate screening procedure for the Δ(*GJB6*-D13S1830) mutation of *GJB6*, as it has been estimated that this deletion could be the cause of non-syndromic autosomal recessive deafness in 15 – 35% of all cases (Del Castillo *et al.*, 2003).

CHAPTER 5: CONCLUSIONS

Hearing loss is the most common inherited sensory disorder (Kelley *et al*, 1998). It has been estimated that 80% of all congenital inherited deafness cases are the result of autosomal recessive inheritance or sporadic mutational events (Estivill *et al.*, 1998). It was decided to conduct a study on the role of *GJB2* as the cause of deafness in South Africa since studies have indicated that mutations involving this gene, which encodes Cx26, is a major contributor to autosomal non-syndromic recessive deafness (Antoniadi *et al.*, 2000). It has been estimated that the screening for two specific mutations, 35delG and 167delT of *GJB2* is expected to diagnose up to 50% of familial and 10 - 40% of sporadic cases in Caucasian populations of European descent (Estivill *et al.*, 1998; Tekin *et al.*, 2001). The information generated in this study would be useful in setting up a population specific diagnostic and counselling program for autosomal non-syndromic deafness in South Africa as no such study has been conducted involving this population.

The first aim of the study was to investigate the entire coding region of *GJB2* to identify any mutations that result in non-syndromic autosomal recessive deafness in the Caucasian and Mixed Ancestry populations of South Africa. This was achieved by the PCR amplification of the entire coding region and subsequently, SSCP analysis and automated DNA sequencing.

In total seven different non-syndromic autosomal recessive deafness mutations and five benign polymorphisms were detected within *GJB2* during screening of 44 families belonging predominantly to the Caucasian and Mixed Ancestry populations of South Africa. The mutations that were detected included the common Caucasian mutation (35delG), other mutations that have been previously reported (312del14, W24X, M34T, V37I and W44X), as well as a novel mutation (N62I). The four benign polymorphisms that were detected have all been reported previously and include V27I, A40A, R127H and V153I. The 167delT mutation of *GJB2* was not detected in this study. This was expected as it has been shown that this mutation is specific to Ashkenazi Jews and in this study group there were no individuals from this genetic background. This therefore reflects that every population should be screened for population specific mutations and the ancestral background of the individuals being screened should also be taken into consideration.

The second aim of the study was to investigate the entire coding region of *GJB6* to identify any mutations that result in non-syndromic autosomal recessive deafness in the Caucasian and Mixed Ancestry populations of South Africa. This was achieved by the PCR amplification of the entire

region as a single fragment that was analysed by automated DNA sequencing. Screening for the Δ(*GJB6*-D13S1830) mutation was attempted using a simple PCR technique that included agarose gel electrophoresis to identify those individuals that were possibly homozygous, heterozygous or wild type normal for the large deletion that involved the *GJB6* gene.

Only a single benign polymorphism was detected in *GJB6* during mutation analysis. The polymorphism, S199T, has been reported before. It has been previously noted that not many mutations causing non-syndromic autosomal recessive deafness have been identified in *GJB6*, therefore it appears that this gene is not as susceptible to the occurrence of mutations as compared with *GJB2*, which has more than 70 reported mutations (Connexin Homepage, 2003). Another possible explanation for the low number of mutations identified to date within *GJB6* could be due to potential lethality in the embryo. This phenomenon has been reported for *GJB2* knockout mice and it is assumed the same may possibly occur for *GJB6* knockout mice, since both genes are highly similar in structure. However, this lethality may carry over to humans for *GJB6* and not for *GJB2* (Kelley *et al.*, 1999).

During the screening for the Δ(*GJB6*-D13S1830) mutation a number of questions arose that concerned the accuracy of the method used. Due to this, some of the results that had been obtained during this study were excluded. The specificity of the primers used was brought into question, as it was noted during NCBI database analysis that the sequences of the primers were found in a number of places throughout the human genome. Specifically the forward primer, GJB6-1R, could possibly result in non-specific amplification as it is found in the forward and reverse complement orientation on chromosome 13. Also during NCBI database analysis it was discovered that the fragments obtained during PCR amplification were in fact localised throughout the genome on various chromosomes as the sequence is possibly that of a genome-wide repeat, known as a LINE. Finally, a recent report clarified the fact that only the deletion of approximately 309 kb exists and not a smaller deletion of roughly 140 kb (Del Castillo *et al.*, 2003). This information influenced our results as it was assumed that the South African population harboured the smaller deletion of 140 kb. Therefore, it was decided that a positive control, containing the Δ(*GJB6*-D13S1830) mutation, was required. Inquires have been made for obtaining such a sample from studies previously conducted within other countries. One such sample has been obtained and it will be used to optimise the PCR conditions for Δ(*GJB6*-D13S1830) mutation screening. Further verification may be conducted with Southern blotting (Del Castillo *et al.*, 2002), depending on the results obtained during the analysis of the South African population with the PCR technique. This work will form

part of the ongoing study of non-syndromic autosomal recessive deafness in the South African population.

The third aim of this study was to analyse the effectiveness of three SSCP gel electrophoresis systems in the detection of mutations in *GJB2*. Three systems that were chosen included a mini PAGE, a SSCP-urea gel electrophoresis and a two buffer gel electrophoresis procedure. It was determined from the results that none of the SSCP gel electrophoresis techniques were effective, because none could detect the most common mutation, 35delG, of *GJB2*. A possible reason for this could be that the fragment size screened was too large, as the sensitivity of SSCP analysis decreases with the increase of fragment sizes over 200 bp. The size of this fragment was 350 bp, and this could have influenced results obtained. Also the position of the mutation within the fragment can also influence the sensitivity of the method. The 35delG mutation lay within the first 40 bp of fragment 1, therefore the SSCP method may detect this mutation successfully if it lay within the middle of the fragment instead. With regards to the detection of other mutations it was determined that the two buffer system was the most effective at detecting mutations within fragment 2 as it clearly demonstrated the 312del14 mutation. Another reason for the two buffer system being regarded as the most efficient system is that more samples can be screened per experiment. This, therefore, means that more samples can be screened per day, which is a great advantage for a diagnostic program.

In the present study optimisation of the different SSCP gel electrophoresis systems did not occur as it was decided that it was necessary to first determine which mutations were the most common to the South African populations. Therefore, a future study could be conducted whereby the different SSCP gel electrophoresis systems could be optimised to specifically detect the common mutations. It is believed that with specific optimisation the different SSCP gel electrophoresis systems will be able to detect the 35delG mutation of *GJB2* as it has been shown to be possible in other studies that have been conducted elsewhere (Kelley *et al.*, 1998). This therefore, should give a more accurate assessment as to whether the two buffer gel electrophoresis system is in fact the best suited for use in a large scale diagnostic program of non-syndromic autosomal recessive deafness. It is still debatable as to whether the SSCP gel electrophoresis system should be used in a screening program, as it is not a completely sensitive technique even when it is fully optimised because novel mutations may still be missed. The percentage of patients that do not contain the previously reported mutations will have to be taken into account. This will determine the possible number of samples that will have to undergo further screening using automated DNA sequencing to determine the deafness causing mutations present. If DNA sequencing technology becomes more cost effective in

the future it could become the method of choice for mutation screening. On the other hand DNA chips may be designed to contain the most common mutations and thereby provide a reliable and time effective method for screening for a large number of mutations in a single reaction.

The fourth and final aim of the study was to determine the allele frequencies of those mutations detected in *GJB2* and *GJB6* amongst the Caucasian and Mixed Ancestry populations of South Africa. This information indicates which mutations are common in these populations and therefore can be used in the establishment of a diagnostic program in the future.

From the results obtained it was determined that the most common mutations amongst the familial study group was the *GJB2* mutation 35delG. The 312del14 of *GJB2* was also common in this group. With regards to the sporadic study group the 312del14 and 35delG mutations were observed at a high frequency. Therefore, in total the most common mutation that was detected within the South African population was the 35delG mutation. To determine if there were different mutations relating to specific South African populations, the result were further analysed for the specific population groups. It was observed that the 35delG mutation was the most common in both the Caucasian and Mixed Ancestry populations. However, the 35delG mutation was more common in the Caucasian population than in the Mixed Ancestry group. This result was expected since the Caucasian population of South Africa is of European descent and the Mixed Ancestry group descends from the Khoi San, Malay, African and Caucasian populations. Interestingly, the Cx26 mutation, 312del14, was only observed in the Caucasians and not in the Mixed Ancestry population. This is also the first time that this mutation has been reported at such a high frequency, which could possibly be due to a founder gene effect. The frequency of the different polymorphisms that were observed in *GJB2* and *GJB6* was also determined and compared to those detected amongst the controls. However, the sample size was relatively small which resulted in the frequency being insignificant. The success rate of mutation detection was also determined (35 %), which can be considered as relatively successful since it has been reported that between 10 – 50% of all the cases with non-syndromic autosomal recessive hearing loss have at least one mutant allele remaining unidentified when only *GJB2* mutation screening is performed (Del Castillo *et al.*, 2003). This therefore indicates that if a diagnostic program for autosomal recessive non-syndromic deafness were to be put in place, focusing only on the mutations of *GJB2* and *GJB6*, a high rate of success in identification of the causative mutations would not be achieved. However, if screening for the Δ(*GJB6*-D13S1830) mutation were included it could lead to an increase in sensitivity by 17 – 34% (Del Castillo *et al.*, 2003).

Future studies should include other population groups of South Africa, such as the different African tribes as well as the South African Indian population. This will mean increasing the sample collection area to include the other provinces of the country instead of just focusing predominantly on the Western Cape province as was done in this study. For this to be achieved, better co-operation and understanding about the heritability of non-syndromic autosomal recessive deafness and the potential benefits of the project will have to be communicated between the researchers and the deaf community so that a sufficient number of samples can be collected.

The inclusion of additional individuals and population groups will lead to a better understanding of the role that *GJB2* and *GJB6* play in South Africa. Another possible area of study would be to determine whether the common mutations that have been observed in the South African populations are derived from a common ancestor. This could be achieved with the use of haplotype studies that would include various polymorphic markers that surround *GJB2* and *GJB6*.

The study could be expanded further by the inclusion of other genes, such as *GJA1* (Cx43) and *GJB3* (Cx31), since it has been reported that these genes are also involved in non-syndromic hearing loss (Liu *et al.*, 2000; Liu *et al.*, 2001). This is important, since it has been shown that mutations in *GJA1* play a role in non-syndromic autosomal recessive deafness amongst the African population of South Africa (Kabahuma *et al.*, 2003). This should help in the identification of the mutations that lead to non-syndromic autosomal recessive deafness in those unexplained cases. Eventually, this knowledge would further improve the impact of a population specific diagnostic program for the detection of non-syndromic autosomal recessive hearing loss in South Africa.

With regards to the establishment of a diagnostic and genetic counselling program for non-syndromic autosomal recessive deafness it is recommended that:

1. Further research be conducted to optimise the various SSCP gel electrophoresis systems so that the most efficient system can be determined. The optimisation of the systems should focus on common mutations, which include the *GJB2* mutations 35delG and 312del14. Conversely, research could be conducted into other screening techniques that are possibly more efficient at detecting mutations and also cost effective.
2. Future studies should include other population groups of South Africa, such as the various African groups as well as the South African Indian population, thereby improving the diagnostic and counselling service that is offered to all populations in the country.

3. The sample size of the various study groups should be increased so that a more accurate frequency of the different mutations related to non-syndromic autosomal recessive deafness in South Africa can be determined. This will again lead to a more accurate diagnostic and genetic counselling service being offered.
4. In screening, patient ethnicity and cultural background should be considered since this will provide a more time effective screening program because population specific mutations can be focused on initially. It will also allow for more accurate risk assessment that is given to the family with non-syndromic autosomal recessive deafness.
5. Other genes that lead to non-syndromic autosomal recessive deafness should also be studied. These genes would include *GJA1* (Cx43) and *GJB3* (Cx31) as they have been reported to play a role in this disorder. Analysis of these genes could then possibly be included into the diagnostic screening when the identification of *GJB2* and/or *GJB6* mutations have failed to provide an answer as to the cause of non-syndromic autosomal recessive deafness within a family or individual.

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APPENDIX A: INFORMED CONSENT

A.1 ENGLISH INFORMED CONSENT FORM

PROJECT HUMAN GENETICS AND AUDIOLOGY (DEPT. ENT) TYGERBERG HOSPITAL: MOLECULAR ANALYSIS OF CONNEXIN 26 AND CONNEXIN 30 MUTATIONS IN NON-SYNDROMIC HEREDITARY DEAFNESS IN SOUTH AFRICA.

Congenital deafness affects 1/1 000 live births and 50% of this is hereditary. 70 - 80% of hereditary deafness in non-syndromic (i.e. only the inner ear affected). Forty-five chromosomal loci have been identified in familial non-syndromic deafness. Hearing loss has been studied through linkage studies in families. *GJB2* gene mutations are amongst those genes associated with non-syndromic deafness. The *GJB2* encodes a gap-junction protein, connexin 26, which is expressed in the inner ear and is important in maintaining cochlea potential. DFNB1 is localised on chromosome 13q11-12. In the Mediterranean European population a single mutant allele of *GJB2* accounts for most of the autosomal recessive deafness defects. The mutation is referred to as 35delG (or 30delG) since it is the deletion of a single nucleotide in a string of 6 guanine residues, beginning at nucleotide position 30 and ending at position 35. In the Ashkenazi Jewish population a 167delT frameshift mutation was commonly found.

Material and methods:

Patients: Families and affected sporadic cases referred from - Carl du Toit Centre, schools for the deaf/ hearing disorders, other referrals. All families entered - 2 unaffected parents and at least 2 affected siblings.

Prerequisite: Consent and audiology of parents and children

Divided in groups for analysis (if enough patients): Group 1 - European extraction

Group 2 - Other

Reason - estimate frequencies of specific mutations and identify possible differences in order to optimise mutation testing.

Test:

Audiology, Blood test - 5-10 ml of EDTA blood for molecular analysis (1-2 teaspoon/s), DNA testing, Pedigree

Outcome:

Patients will be informed of the outcome of the study. Eventually one hopes to find a high frequency of a specific mutation, in order to be of use to parents who have had one child with non-syndromic neurosensory deafness to test whether this is a sporadic condition or has an increased recurrence risk.

Cost:

No cost for entering, pedigree, blood tests or parental audiograms. We cannot, however, pay you for inclusion either.

Ethics:

No results will be published that would identify any person, but accumulated results may be published in a medical journal.

DECLARATION:

I,.....the undersigned, [ID .....]
the patient or in my capacity as .....of patient.....
[ID.....] of .....(address).

I declare that:

1. I/the patient was invited to participate in the above mentioned research project which is being undertaken by the Division of Human Genetics of the University of Stellenbosch.
2. It has been explained to me that

2.1 The project is being undertaken to investigate the role of connexin 26 and connexin 30 mutations as a cause of deafness in the South African population.

2.2 If I/the patient participate(s) in the project, only 20-50 ml of blood will be collected from the arm. The plasma and DNA/RNA extracted from the blood will be used in laboratory tests. Index patients and family members will be included in follow-up studies (e.g. blood sampling, laboratory testing, magnetic resonance imaging and completion of questionnaires).

2.3 A blood sample will be required from both affected and unaffected family members. The project should not take more than 2 years.

2.4 If the genes are found associated with non-syndromic deafness in the South African population, prenatal testing may be necessary in future.

3. I/the patient have/has been warned that the drawing of blood may result in slight discomfort, which can be coupled with bleeding where the needle pierces the skin.
4. It has been explained to me/the patient that participation in this project will result in the broadening of medical knowledge. The possible development of an accurate diagnostic DNA test for the condition(s) might lead to early detection and preventative treatment of affected individuals and improved counselling and risk assessment of families.
5. I/the patient have/has been informed that all information collected will be treated confidentially. The results will be used for publication in scientific journals/theses, without revealing the identity of any individual.
6. I/the patient may, during or on completion of the project, request the results of the tests without any conditions attached thereto, since the results could be advantageous to me/the patient and my/his family. Genetic counselling will be provided with this information.
7. I/the patient have/has been told that participation is voluntary and that I/the patient may refuse to participate in this project and that I/the patient may also at any time withdraw my/his or her participation from the project. Refusal or withdrawal from the project will in no way affect my/his or her present or future treatment at this institution. I/the patient also understands that the researcher may withdraw me/him or her from the project if he/she considers it in my/the patient's best interest.
8. I/the patient was not pressurised to participate in this project and I/the patient know that I/the patient may at any time withdraw from this project without penalisation.
9. Participation in this project will not result in unnecessary expenses for me/the patient.
10. **I/the patient voluntarily agree(s) to** participate in the above mentioned project

SIGNED/AFFIRMED AT.....ON.....20.....

....

Signature of patient/ representative of patient Witness

Forward blood sample collected in EDTA or ACD-containing tubes (5-20 ml) to the following address to reach the laboratory within one week:

Dr G de Jong

Division of Human Genetics

Faculty of Health Sciences

Francie van Zijl Drive

Room 2126 / PO Box 19063

Tygerberg 7505

Tel (021) 9389218

DECLARATION BY OR ON BEHALF OF THE RESEARCHER

I,.....declare that I

1. have explained the information in this document to the patient, ...and/or his/her representative.
2. Requested him/her/them to ask questions where anything was unclear.

Dr/Mr/Ms.....

SIGNED AT.....ON.....20....

....

Researcher/Research representative Witness

IMPORTANT INFORMATION

Dear Patient/Representative of the patient

Thank you for participating in this project. If at any time during the course of the project

1. An emergency situation arises resulting from the research, or
2. You require further information regarding the project, or
3. If any of the following occurs, please contact **Dr. G de Jong** at telephone number: **(021) 9389218**
   (a) The condition of the participant suddenly deteriorates
   (b) An apparent unaffected family member suddenly becomes ill or dies
   (c) A participant changes address and/or telephone number.

A.2 AFRIKAANS INFORMED CONSENT FORM

PROJEK VAN MENSGENETIKA AFDELING EN OUDIOLOGIE (DEPT. ONK) TYGERBERG HOSPITAL: CONNEXIN 26 EN CONNEXIN 30 MUTASIES IN NIE SINDROMIESE DOOFHEID IN DIE SUID AFRIKAANSE POPULASIE.

Aangebore doofheid affekteer ongeveer 1/1 000 lewendgeborenes en 50% hiervan is oorerflik. 70-80% van oorerflike doofheid is nie sindromies d.w.s. slegs die binne-oor is geaffekteer. 45

verskillende chromosomale loci is al geïdentifiseer in families met nie sindromiese doofheid. Gehoorgestremdheid is tot nou toe bestudeer deur genetiese koppeling studies in families. Die *GJB2* geen kodeer vir 'n proteien, connexin 26 wat uitgedruk word in die binne-oor en is belangrik in die behoud van kogliêre potensiaal. Hierdie DFNB1 geen is gelokaliseer op chromosoom 13q11-12. Mutasies in hierdie *GJB2* geen word geassosieer met nie sindromiese doofheid. In die Mediterieense populasie vind ons dat 40% van die outosomaal resessieve doofheid geassosieer is met 'n enkele mutasie in die *GJB2* geen. Hierdie mutasie is bekend as die 35delG (of 30delG) aangesien dit 'n enkele mukleotied in 'n reeks van 6 guanien residus, wat begin by die nukleotied in posisie 30 en eindig by posisie 35. In die Ashkenazi Joodse populasie is daar 'n ander delesie wat algemeen is nl., 167delT.

Metodes wat gebruik sal word:

Pasiënte: Families en geaffekteerde sporadiese gevalle wat verwys word van o.a. Carl du Toit Sentrum, skole vir dowes of gehoorgestremdes en ander verwysings.

Families betrek: die wat twee ongeaffekteerde ouers het en minstens twee aangetaste kinders.

Voorvereistes: toestemming van ouers

oudiologiese evaluasie van ouers en kinders

Die groepe sal in twee verdeel word (as daar genoeg pasiënte): Groep 1 - Europese afkoms

Groep 2 - Ander

Rede hiervoor: die mutasies in die verskillende groepe mag verskil en dit is nodig om die verskille te identifiseer om optimale toetsing te vewerkstellig.

Toetse:

Oudiologie, bloodtoets - 5-10 ml EDTA bloed vir molekulêre analise (1-2 teelepels), DNA toetsing, stamboom.

Uitkoms:

Die pasiënte sal ingelig word oor die resultate van die studie. Die uiteindelike doel van die studie is om hopenlik 'n spesifieke mutasie te kry wat algemeen is in ons populasie wat gebruik kan word in families waar slegs een kind nog aangetas is om uit te sorteer of dit 'n toevallige afwyking is en of daar 'n herhalingsrisiko gaan wees.

Koste:

Geen koste is verbonde aan toetrede tot die studie, stambome opstel, bloedtoetse of ouerlike oudiogramme. Ons kan egter nie vir u betaal vir insluiting by die studie nie.

Etiese oorweginge:

Geen resultate sal gepubliseer word wat enige persoon kan identifiseer nie. Die uiteindelike resultate mag egter moontlik gepubliseer word in 'n mediese joernaal om tot nut te wees van ander mense.

VERKLARING DEUR OF NAMENS PASIëNT

Ek, die ondergetekende,.....[ID.....]
die pasiënt of in my hodanigheid as.....van die pasiënt.....
[ID.....] van.....(adres)

Ek bevestig dat:

1. Ek/die pasiënt uitgenooi is om deel te neem aan bogemelde navorsingsprojek wat deur die Afdeling Mensgenetika van die Universiteit van Stellenbosch onderneem word.
2. Daar aan my verduidelik is dat

2.1 Die projek onderneem word om die rol van die connexin 26 en connexin 30 mutasies to ondersoek as 'n oorsaak van doofheid in die Suid-Afrikaanse populasie.

2.2 Indien ek deelneem aan die projek, slegs 20-50 ml bloed uit die voorarm versamel sal word. Die plasma en DNA/RNA sal uit die bloed geïsoleer word vir gebruik in laboratorium toetse. Indekspasiënte en hul familielede sal ingesluit word in opvolgstudies (bv. bloedmonsterneming, magnetiese resonans beelding en gevra word om 'n vraelys in te vul).

2.3 Bloedmonsters benodig word van beide geaffekteerde pasiënte en ongeafekteerde familielede. Die projek behoort nie langer as 2 jaar te duur nie.

2.4 Indien die gene wel betrokke is by nie sindromiese doofheid, voorgeboorte analise in die toekoms moontlik kan word.

3. Ek gewaarsku is dat die proses van bloedtrek effense ongemak mag meebring, wat gepaard gaan met bloeding waar die naald die vel binnedring.
4. Daar verder aan my verduidelik is dat deelname aan die projek sal bydra tot die uitbouing van mediese kennis. Die moontlike ontwikkeling van 'n akkurate DNA diagnose van die kondisie(s) wat mag lei to vroeë opsporing en verveterde behandeling van aangetaste persone en verbeterde raadgewing en risiko bepaling in families.
5. Ek meegedeel is dat die inligting wat ingewin word as vertroulik beskou sal word, maar wel aangewend sal word vir publikasies in vaktydskrifte en tesisse.

6. Ek/die pasiënte tydens/na afhandeling van die projek die uitslag van die toetse kan aanvra sonder dat daar enige voorwaardes aan verbonde is, aangesien die kennis tot my en my familie se voordeel sal wees. Genetiese raadgewing gegee sal word saam met die inligting.
7. Ek meegedeel is dat ek mag weier om deel te neem/die pasiënt te laat deelneem aan hierdie projek Iasook dat ek/die pasiënt te enige tyd deelname daaraan mag staak) en dat sodanige weiering of staking nie op enige manier my/die pasiënt se huidige/toekomstige behandeling by hierdie inrigting sal genadeel nie. Ek verstaan ook dat die navorser my/die pasiënt van die projek mag onttek indien dit in my/die pasiënt se belang geag word deur hom/haar.
8. Daar geen dwang op my geplaas is om toe to stem to my/die pasiënt se deelname aan hierdie projek nie en dat ek besef dat ek/die pasiënt deelname te enige tyd mag staak sonder enige penalisasie.
9. Deelname aan die projek geen addisionele koste vir my/die pasiënt inhou nie.
10. **Ek stem hiermee vrywillig in** om deel te neem aan die bogemelde projek/dat die pasiënt deelneem aan die bogemelde projek.

GETEKEN/BEVESTIG TE.....OP.....20.....

....

Pasiënt/verteenwoordiger van pasiënt Getuie

Stuur bloemonster gekollekteer in EDTA buise (5-20 ml) na die onderstaande adres on die laboratorium te bereik binne die bestek van een week:

Dr G de Jong

Afdeling Mensgenetika

Fakulteit Gesonheidwetenskappe

Francie van Ziyl Rylaan

Kamer 2128/ Posbus 19063

Tygerberg 7505

Tel (021) 9389218

VERKLARING DEUR OF NAMENS NAVORSER

Ek,.....verklaar dat ek:

1. Die inligting vervat in hierdie dokument aan die pasiënt.....en/of sy/haar verteenwoordiger.....verduidelik het;

2. Hom/haar/hulle versoek het om vrae aan my te stel indien daar enigiets onduidelik was;

Dr/Mnr/Me.....

GETEKEN TE.....OP.....20.....

....

Navorser/navorser se verteenwoordiger Getuie

BELANGRIKE INLIGTING

Geagte pasiënt/ verteenwoordiger van die pasiënt. Baie dankie vir u/die pasiënt se deelname aan hierdie studie. Indien daar te enige tyd tydens die duur van die projek:

1. 'n noodsituasie onstaan wat spruit uit die navorsing, of
2. u enige verdere inligting aangaande die projek verlang, of
3. die volgende plaasvind moet u asseblief vir **Dr. G de Jong**, was as kontakpersoon sal optree vir nodige verwysing van die pasiënt, kontak by telefoonnommer: **(021) 9389218**
   (a) die siktetoestand van 'n deelnemer aan die projek skielik versleg
   (b) 'n oënskynlik onaangetaste familielid skieik siek word of sterf
   (c) 'n deelnemer aan die projek van adres/telefoon nommer verander

APPENDIX B: RESEARCH PRESENTATIONS

Results presented in this thesis were presented at the following international and national conferences. The presenting author's name is underlined in each case.

B.1 RESEARCH PRESENTED AT INTERNATIONAL CONFERENCES

52nd ANNUAL MEETING OF THE AMERICAN SOCIETY OF HUMAN GENETICS: Baltimore, Maryland, USA, October 2002.

de Jong G., Whitehead C., Muller L., du Plessis L., Kotze M. and Warnich L. (*GJB2*) Connexin 26 and 30 (*GJB6*) mutations in a South African population with non-syndromic deafness of probable autosomal recessive origin.

B.2 RESEARCH PRESENTED AT NATIONAL CONFERENCES

10TH BIENNIAL CONGRESS OF THE SOUTHERN AFRICAN SOCIETY OF HUMAN GENETICS: Durban, South Africa, May 2003.

Whitehead C., de Jong G., Muller L., Kotze M.J. and Warnich L. Non-syndromic autosomal recessive deafness and the role that connexin 30 mutations play in the South African population.

10TH BIENNIAL CONGRESS OF THE SOUTHERN AFRICAN SOCIETY OF HUMAN GENETICS: Durban, South Africa, May 2003.

de Jong G., Whitehead C., Muller L.M., Kotze M.J. and Warnich L. Autosomal recessive deafness and connexin 26 and 30 (*GJB2* and *6* gene mutations) in a South African population.

CAPE BIOTECH: Somerset West, Cape Town, South Africa, November 2002.

Whitehead C., de Jong G., Muller L., Kotze M.J. and Warnich L. Molecular diagnostics on non-syndromic hereditary deafnes in South Africa: Study of connexin 26 and connexin 30 mutations.

B.3 RESEARCH PRESENTED IN THE DEPARTMENT OF GENETICS, UNIVERSITY OF STELLENBOSCH

SEMINAR DAY 2003: University of Stellenbosch, Stellenbosch, South Africa, June 2003.

Whitehead C., de Jong G., Muller L., Kotze M.J. and Warnich L. Connexin mutations and the role they play in the deaf population of South Africa.

SEMINAR DAY 2002: University of Stellenbosch, Stellenbosch, South Africa, June 2002.
Whitehead C., de Jong G., Muller L., Kotze M.J. and Warnich L. Childhood hereditary deafness in the South African population.

APPENDIX C: MUTATION ANALYSIS RESULTS

This appendix contains the results obtained during the mutation screening of *GJB2* of both the familial and sporadic study groups.

C.1 FAMILIAL CASES

Family Number	Ethnic Group	Sample Number	Family Relation	Audiology Results	*GJB2* Mutation Analysis Results
1	Caucasian	6045/99A	Father	Normal	Wild Type/Wild Type
		6045/99B	Mother	Normal	Wild Type/Wild Type
		6046/99C	Daughter	Deaf	Wild Type/Wild Type
		6045/99	Son	Deaf	Wild Type/Wild Type
2	Caucasian	6078/99	Father	Normal	35delG/Wild Type
		6079/99	Mother	Normal	N62I/Wild Type
		6865/99	Son	Deaf	35delG/N62I
		6080/99	Daughter	Deaf	35delG/N62I
3	Caucasian	83/00	Mother	Normal	Wild Type/Wild Type
		82/00	Father	Normal	Wild Type/Wild Type
		85/00	Son	Deaf	Wild Type/Wild Type
		84/00	Daughter	Deaf	Wild Type/Wild Type
4	Caucasian	87/00	Mother	Normal	Wild Type/Wild Type
		89/00	Son	Normal	Wild Type/Wild Type
		88/00	Son	Deaf	Wild Type/Wild Type
		91/00	Daughter	Normal	Wild Type/Wild Type
		90/00	Daughter	Deaf	Wild Type/Wild Type
5	Caucasian	623/00	Mother	Normal	312del14/Wild Type
		624/00	Son	Deaf	35delG/312del14
		625/00	Son	Deaf	35delG/312del14
6	Caucasian	616/00	Mother	Normal	35delG/Wild Type
		617/00	Son	Deaf	35delG/312del14
		618/00	Daughter	Deaf	35delG/312del14
7	Caucasian	619/00	Father	Normal	Contaminated Sample
		620/00	Mother	Normal	Wild Type/Wild Type
		621/00	Daughter	Deaf	Wild Type/Wild Type
		622/00	Daughter	Deaf	Wild Type/Wild Type
8	Caucasian	615/00	Son	Deaf	Wild Type/Wild Type
9	Caucasian	8/01	Father	Normal	35delG/Wild Type
		9/01	Mother	Normal	35delG/Wild Type
		10/01	Daughter	Deaf	35delG/35delG
		11/01	Daughter	Deaf	35delG/35delG
		12/01	Daughter	Normal	Wild Type/Wild Type
10	Caucasian	29/01	Mother	Normal	312del14/Wild Type
		30/01	Daughter	Deaf	312del14/312del14
		31/01	Son	Deaf	312del14/312del14
11	Caucasian	53/01	Mother	Normal	35delG/Wild Type
		54/01	Father	Normal	312del14/Wild Type
		55/01	Daughter	Deaf	35delG/312del14
		56/01	Son	Deaf	35delG/312del14
12	Mixed Ancestry	230/01	Daughter	Deaf	35delG/35delG
		231/01	Daughter	Deaf	35delG/35delG
13	Mixed Ancestry	229/01	Son	Deaf	Wild Type/Wild Type
		228/01	Son	Deaf	Wild Type/Wild Type

14	Caucasian	541/01	Father	Normal	312del14/Wild Type
		542/01	Mother	Normal	312del14/Wild Type
		543/01	Son	Deaf	312del14/312del14
		544/01	Son	Deaf	312del14/312del14
15	Caucasian	505/01	Father	Normal	Wild Type/Wild Type
		506/01	Mother	Normal	Wild Type/Wild Type
		507/01	Son	Deaf	Wild Type/Wild Type
		508/01	Son	Deaf	Wild Type/Wild Type
16	Caucasian	F16.1	Son	Deaf	Wild Type/Wild Type
		F16.2	Son	Deaf	Wild Type/Wild Type

C.2 SPORADIC CASES

Family Number	Ethnic Group	Sample Number	Family Relation	Audiology Results	*GJB2* Mutation Analysis Results
1	Caucasian	502/00	Mother	Normal	Wild Type/Wild Type
		501/00	Daughter	Deaf	35delG/Wild Type
2	Caucasian	43/01	Daughter	Deaf	35delG/312del14
		44/01	Mother	Normal	35delG/Wild Type
		46/01	Father	Normal	312del14/Wild Type
3	Caucasian	67/01	Mother	Normal	312del14/Wild Type
		68/01	Son	Deaf	35delG/312del14
4	Caucasian	225/01	Mother	Normal	Wild Type/Wild Type
		224/01	Father	Normal	Wild Type/Wild Type
		226/01	Son	Deaf	Wild Type/Wild Type
5	Caucasian	5.3	Father	Normal	Wild Type/Wild Type
		247/01	Mother	Normal	Wild Type/Wild Type
		246/01	Son	Deaf	Wild Type/Wild Type
6	Caucasian	312/01	Father	Normal	Wild Type/Wild Type
		313/01	Mother	Normal	Wild Type/Wild Type
		314/01	Son	Deaf	Wild Type/Wild Type
7	Caucasian	434/01	Father	Normal	312del14/Wild Type
		435/01	Mother	Normal	Wild Type/Wild Type
		436/01	Daughter	Deaf	312del14/Wild Type
9	Mixed Ancestry	s9.1	Father	Normal	W24X/Wild Type
		s9.2	Mother	Normal	35delG/Wild Type
		s9.3	Daughter	Deaf	35delG/W24X
		s9.4	Daughter	Deaf	Identical twin of 9.3
10	Indian	s10.1	Mother	Normal	Wild Type/Wild Type
		s10.2	Son	Deaf	Wild Type/Wild Type
		Family also has the V153I polymorphism of GJB2			
11	Indian	s11.1	Father	Normal	Wild Type/Wild Type
		s11.2	Mother	Normal	Wild Type/Wild Type
		s11.3	Son	Deaf	Wild Type/Wild Type
12	Caucasian	s12.1	Father	Normal	Wild Type/Wild Type
		s12.2	Mother	Normal	312del14/Wild Type
		Family also has the V153I polymorphism of GJB2			
13	Caucasian	s13.1	Father	Normal	Wild Type/Wild Type
		s13.2	Mother	Normal	Wild Type/Wild Type
		s13.3	Son	Deaf	Wild Type/Wild Type
14	African	s14.1	Son	Deaf	Wild Type/Wild Type
15	African	s15.1	Son	Deaf	Wild Type/Wild Type
16	African	s16.1	Daughter	Deaf	Wild Type/Wild Type

17	Caucasian	s17.1	Father	Normal	35delG/Wild Type
		s17.2	Mother	Normal	35delG/Wild Type
		s17.3	Daughter	Deaf	35delG/35delG
18	Mixed Ancestry	s18.1	Father	Normal	Wild Type/Wild Type
		s18.2	Mother	Normal	Wild Type/Wild Type
		s18.3	Daughter	Deaf	Wild Type/Wild Type
19	Caucasian	s19.1	Mother	Normal	Wild Type/Wild Type
		s19.2	Son	Deaf	Wild Type/Wild Type
20	Caucasian	s20.1	Father	Normal	312del14/Wild Type
		s20.2	Mother	Normal	312del14/Wild Type
		s20.3	Son	Deaf	312del14/312del14
21	Indian	s21.1	Father	Normal	V37I/Wild Type
		s21.2	Mother	Normal	Wild Type/Wild Type
		s21.3	Son	Deaf	V37I/Wild Type
22	Mixed Ancestry	s22.1	Mother	Normal	Wild Type/Wild Type
		s22.2	Daughter	Deaf	Wild Type/Wild Type
23	Caucasian	s23.1	Father	Normal	Wild Type/Wild Type
		s23.2	Mother	Normal	Contaminated DNA
		s23.3	Daughter	Normal	Wild Type/Wild Type
		s23.4	Son	Deaf	Wild Type/Wild Type
24	Caucasian	s24.1	Mother	Normal	Wild Type/Wild Type
		s24.2	Daughter	Deaf	Wild Type/Wild Type
25	Caucasian	s25.1	Father	Normal	Wild Type/Wild Type
		s25.2	Mother	Normal	Wild Type/Wild Type
		s25.3	Daughter	Deaf	Wild Type/Wild Type
26	Caucasian	s26.1	Daughter	Deaf	312del14/Wild Type
27	Caucasian	s27.1	Mother	Normal	Wild Type/Wild Type
		s27.2	Father	Normal	Wild Type/Wild Type
		s27.3	Daughter	Deaf	Wild Type/Wild Type
28	Mixed Ancestry	s28.1	Father	Normal	Wild Type/Wild Type
		s28.2	Mother	Normal	Wild Type/Wild Type
		s28.3	Daughter	Deaf	Wild Type/Wild Type
29	Caucasian	s29.1	Father	Normal	Wild Type/Wild Type
		s29.2	Mother	Normal	Wild Type/Wild Type
		s29.3	Son	Deaf	Wild Type/Wild Type